Biomechanical and molecular characterisation of Mycoplasma bovis and related species

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Biochemical and molecular characterisation of *Mycoplasma bovis* and related species

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Abstract

Mycoplasma agalactiae and *M. bovis* are closely related and were originally classified within the same species. *M. agalactiae* is the principal cause of contagious agalactia in sheep and goats, which is widespread in Southern Europe and of major economic importance. *M. bovis* associated with mastitis, arthritis, pneumonia, and genital and ocular lesions in cattle, is of increasing importance in Europe and is endemic in most parts of the UK. Relatively little is known of their metabolism though they do not ferment glucose or hydrolyse arginine. The initial aims of the project were to determine the patterns, kinetics and regulation of energy substrate utilisation by field isolates and laboratory strains of both species. During this work, *M.* ovine serogroup 11, a cause of infertility in sheep, was isolated in the UK for the first time and isolates were also included in the study. *M. bovigenitalium,* which causes reproductive disorders, infertility and impaired sperm motility, appears biochemically similar to *M. agalactiae* and *M. bovis.*

Substrate oxidation by 54 strains of *M. agalactiae*, *M. bovis*, *M. bovigenitalium* and *M.* ovine serogroup 11 was determined from the reduction in dissolved oxygen tension. All strains of the four species were characterised by: a high affinity for L-lactate, 2-oxobutyrate, pyruvate and isopropanol; ability to oxidise acetaldehyde, ethanol and propanol, and an inability to metabolise some sugars, glycerol, amino acids and tricarboxylic acid intermediates. Oxidisable organic acids were metabolised at relatively high rates and with high affinity. Isopropanol was metabolised with high rate and high affinity, and its oxygen uptake was consistent with oxidation to acetone. It is assumed that the principal role of alcohol dehydrogenase is the oxidation of isopropanol. The intermediate product of ethanol oxidation is acetaldehyde, which was oxidised to acetate by representative strains of all the species. These strains appear entirely dependent upon organic acids for their energy generation and their growth yield was markedly increased by the addition of 2-oxobutyrate and pyruvate. Although isopropanol was oxidised at high rates, it did not act as an energy source.

PRM medium modified by using novel vegetable peptones (instead of ruminant peptones) gave high growth yields. Pyruvate was confirmed their energy source and glucose was omitted. PRM medium for the growth of these organisms was made

selective by the addition of the inhibitors (α-methyl glucoside, L-citrulline, L-lysine, L-ornithine and penicillin) of fermentative and arginine hydrolying mycoplasmas.

The distinctive pattern of substrate oxidation by non-fermentative and non-arginine hydrolysing mycoplasmas enabled their differentiation from other groups such as *M. mycoides* SC, which were characterised by: an inability to metabolise maltose and trehalose; a low affinity for glucosamine and mannose; a high affinity for glucose, fructose and N-acetylglucosamine, and an ability to oxidise lactate, pyruvate and 2-oxobutyrate. No European SC strains were able to oxidise glycerol while African SC strain (SH9) oxidised glycerol and showed different kinetics for glucose oxidation.

All strains studied possessed glycerol kinase activity producing L-α-glycerophosphate (GP), but they lacked GP oxidase activity. Hydrogen peroxide (H_2O_2) production from NADH oxidation by lysed cells varied amongst all strains studied in this thesis and were further grouped into high and low H_2O_2-producing strains. There were substantial differences in the production of H_2O_2 e.g. in *M. bovis* the amount of H_2O_2 produced decreased during repeated *in vitro* passage. After the 50^{th} and the 200^{th} passage H_2O_2 was reduced to approx. 50 % and 7 % respectively. SDS-PAGE analysis of these passaged mutants revealed one band (28 kDa) was lost. Passaging *Mycoplasma* strains *in vitro* may reduce their virulence.

The ability of more than twenty *Mycoplasma* species to hydrolyse fatty acid esters was tested. Colonies of *M. agalactiae* and *M. bovis* became bright red (within 30 mins) after application of SLPA-octanoate. In contrast, strains of other test species did not give colour after more than five hours incubation. A rapid qualitative and quantitative chromogenic assay was developed for identification of *M. agalactiae* and *M. bovis* based on their distinctive ability to hydrolyse a novel chromogenic substrate (4[2-(4-octanoyloxy-3, 5-dimethoxy phenyl)-vinyl]-quinolinium-1-(propan-3-yl carboxylic acid) bromide; SLPA-octanoate). Lipolytic activity was detected by a fluorimetric method and it was also detected on native gels.

Type and field isolates of *M. bovis* were characterised by restricted fragment length polymorphism (RFLP), restriction endonucleases, immunoblotting, SDS-PAGE and pulsed field gel electrophoresis (PFGE). RFLP and SDS-PAGE gave identical banding

patterns while immunoblotting and PFGE gave discriminatory banding patterns. SDS-PAGE analysis revealed no difference within the species but differences were observed between species. Information obtained in this thesis should be of value for epidemiological studies and provide insights into the pathogenicity of the mycoplasmas and can also be used for development of improved culture media and rapid biochemical tests. It also increased knowledge of mycoplasma physiology and metabolism.

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Table of contents

	Title	I
	Dedication	II
	Abstract	III
	Acknowledgements	VI
	Table of contents	VII
	List of Tables	XII
	List of Figures	XIII
	List of Abbreviations	XVII

Chapter 1

1.	**Introduction**	1
1.1	Mollicutes	1
1.2	Classification of mollicutes	2
1.3	Genus *Mycoplasma*	3
1.3.1	*Mycoplasma agalactiae* and *Mycoplasma bovis*	9
1.4	Morphology and cell division	13
1.5	Colony shape	15
1.6	Ultrastructure	15
1.7	Membrane structure	16
1.8	Pathogenicity	17
1.9	Adhesion	19
1.9.1	Variable surface proteins	20
1.10	Oxidative damage	21
1.11	Toxin production	25
1.12	Capsule production	26
1.13	Enzyme activities	27
1.14	Growth requirements and growth media	29
1.15	Energy sources	32
1.16	Transport systems	36
1.17	Identification of mollicutes, assignment of families, genera and species	40
1.18	Aims of the project	42

Chapter 2

2.	**Materials and methods**	44
2.1	Organisms used in the study	44
2.2	Growth of organisms	44
2.3	Growth media	44
2.3.1	Proteose peptone medium	44
2.3.2	PRM medium	45
2.3.3	Eaton 's medium	45
2.3.4	SP4 medium	45
2.4	Blood agar medium	46
2.5	Fresh yeast extract	53

2.6	Suspension media	53
2.6.1	Ringer HEPES buffer (RH)	53
2.6.2	Normal saline solution	53
2.6.3	Salt I solution	54
2.7	Preparation of cell suspension	54
2.8	Measurement of optical density	54
2.9	Viable count determination	54
2.10	Protein concentration determination	55
2.11	Determination of substrate utilisation	55
2.11.1	Measurement of oxygen uptake and preparation of oxygen electrode	55
2.11.2	Determination of NADH oxidase, glycerophosphate oxidase, and glycerol kinase activities	56
2.11.3	Hydrogen peroxide production by lysed cells and whole cells	57
2.12	Metabolism monitored by pH change	57
2.13	Qualitative and quantitative detection of lipolytic activity in mycoplasmas	58
2.13.1	Preparation of chromogenic substrates	58
2.13.2	Qualitative detection of lipase activity	58
2.13.3	Preparation of *M. bovis* cytoplasmic and membrane fractions for the quantitative analysis of the lipolytic activity	58
2.13.4	Lipase assay using spectrophotometric method	58
2.13.5	Lipase assay using fluorimetric method	60
2.14	Detection of lipolytic activity in *M. bovis* on polyacrylamide gels	60
2.15	Preparation of the *M. bovis* cells for genetic analysis	61
2.15.1	Extraction of DNA	61
2.15.2	DNA quantification	61
2.15.3	Detection of *M. bovis* by PCR	62
2.15.4	Restriction enzymes digestion of PCR product	63
2.16	Restriction enzymes digestion of whole genomic DNA	63
2.17	Comparison of restriction patterns of *M. bovis* by pulsed field gel electrophoresis (PFGE)	63
2.17.1	Preparation of DNA and PFGE analysis	63
2.18	Growth and preparation of the *M. bovis* and other species for protein profile and immunoblotting	64
2.18.1	Harvesting of organisms	64
2.18.2	Preparation of the gels	64
2.18.3	Preparation of the samples and electrophoresis	65
2.18.4	Staining and destaining of the gels	66
2.19	Immunoblotting method	66
2.20	Substrates, enzymes and chemicals used in the study	67

Chapter 3

3.	**Kinetics of substrate oxidation by *M. agalactiae*, *M. bovis*, *M. bovigenitalium* and *M.* ovine serogroup 11**	71
3.1	Introduction	71
3.2	Results and discussion	73
3.2.1	Optimisation and determination of substrate oxidation	73

3.2.2	Determination of affinity constants K_s and V_{max} values	75
3.2.3	Patterns and kinetics of substrate oxidation by *M. agalactiae*, *M. bovis*, *M. bovigenitalium* and *M.* ovine serogroup 11	76
3.2.3.1	Oxidation of organic acids	76
3.2.3.2	Oxidation of alcohols	78
3.2.3.3	Oxidation of sugars	89
3.2.3.4	Oxidation of citric acid cycle intermediates	90
3.3	Substrate utilisation by *M. mycoides* subsp. *mycoides* strains	90
3.3.1	Oxidation of glucose	90
3.3.2	Oxidation of fructose and N-acetylglucosamine	94
3.3.3	Oxidation of glucosamine and mannose	95
3.3.4	Oxidation of maltose and trehalose	96
3.3.5	Oxidation of glycerol	96
3.3.6	Oxidation of organic acids	97
3.4	The effect of metabolisable substrates on the growth of *M. agalactiae*, *M. bovis*, *M. bovigenitalium* and *M.* ovine serogroup 11	98
3.5	The effect of inhibitors on the growth of *M. bovis* and *M.* ovine serogroup 11	114
3.6	Conclusions	115

Chapter 4

4.	**Hydrogen peroxide production by members of *M.agalactiae*, *M. bovis*, *M. bovigenitalium*, *M.* ovine serogroup 11 and *M. mycoides* SC**	122
4.1	Introduction	122
4.2	Results and discussion	125
4.2.1	NADH oxidation and H_2O_2 production by lysed cells of *M. agalactiae, M. bovis, M. bovigenitalium and M.*ovine serogroup 11.	125
4.2.2	H_2O_2 production from oxidation of L-α-glycerophosphate (GP)	132
4.2.3	H_2O_2 production by whole cells metabolising pyruvate, isopropanol and NADH	133
4.2.4	Effect of *in vitro* serial passage of *M. bovis* 119B96 strain on production of H_2O_2.	137
4.2.5	Detection of glycerol kinase activity	140
4.3	H_2O_2 production and NADH oxidation by lysed cells of *M. mycoides* subsp. *mycoides* SC strains.	145
4.4	Conclusions	148

Chapter 5

5.	**Detection of lipolytic activity in mycoplasmas**	150
5.1	Introduction	150
5.2	Results and discussion	153
5.2.1	Development and optimisation of a chromogenic detection system for mycoplasmas	153
5.2.1.1	Importance of colony age	155

5.2.1.2	Substrate specificity	155
5.2.1.3	Solubility of SLPA-esters	155
5.2.1.4	Effect of addition of BSA	156
5.2.1.5	Effect of temperature on plate assay	156
5.2.1.6	Stability of substrate on storage	157
5.2.1.7	Effect of different animal sera and freeze-drying	157
5.2.2	Qualitative lipase assay method	157
5.2.3	Detection of lipolytic activity using chromogenic substrates	157
5.2.4	Development of the field kit	159
5.2.5	Effect of CO_2 on lipase activity	160
5.2.6	Confirmation of lipolytic activity by pH change using glycerol tributyrate substrate	163
5.3	Quantitative detection of lipolytic activity in *Mycoplasma bovis*	163
5.3.1	Comparison of spectrophotometric and fluorimetric lipase assays	163
5.3.2	Development of spectrophotometric lipase assay	163
5.3.3	Spectrophotometric lipase assay	164
5.3.4	Effect of detergents on lipase activity measured by the spectrophotometric assay	165
5.3.5	Effect of culture age on lipase activity measured by the spectrophotometric assay	165
5.3.6	Fluorimetric assay	166
5.4	Detection of lipolytic activity on polyacrylamide gels	171
5.4.1	Detection of lipase activity on SDS-PAGE	171
5.4.2	Detection of lipase activity on native gels	172
5.5	Conclusions	175

Chapter 6

6.	**Determination of relationship amongst type and field isolates of *Mycoplasma bovis* by restricted fragment length polymorphism (RFLP), restriction endonucleases analysis, pulsed field gel electrophoresis (PFGE), sodium dodecyl gel electrophoresis (SDS-PAGE) and immunoblotting**	176
6.1	Introduction	176
6.2	Results and discussion	177
6.2.1	Analysis of *M. bovis* strains by PCR and restricted fragment length polymorphism (RFLP)	177
6.2.2	Restriction enzymes digestion of whole genomic DNA of *M. bovis*	184
6.2.3	Pulsed field gel electrophoresis (PFGE) profiles of *M. bovis*	184
6.2.4	SDS-PAGE analysis of *M. bovis* strains	188
6.2.5	SDS-PAGE analysis of highly passaged *M. bovis* strain 119B96	189
6.3	SDS-PAGE profiles of *M. agalactiae, M. bovis, M. bovigenitalium* and *M.* ovine serogroup11.	192
6.4	Immunoblotting of *M. bovis*	195
6.5	Conclusions	196

Chapter 7

7.	**General discussion**	199
7.1	The development of biochemical procedures and improvement of medium	199
7.2	Study of pathogenicity factors	205
7.3	Correlation of biochemical studies with the RFLP, PFGE, restriction endonucleases, SDS-PAGE and immunoblotting	207
7.4	Future work	209

References 212

Appendices 251

Appendix 1 251

Appendix 2 252

Appendix 3 254

List of publications 255

List of Tables

1.1	General characteristics and taxonomy of the class *Mollicutes*	4
1.2	Mycoplasma diseases of animals	8
1.3	Essential constituents of defined and semi-defined media for *M. mycoides* strain Y and *A. laidlawii* strain B	34
2.1	Organisms used in the study	47
2.2	Oligonucleotide primers for PCR detection of *M. bovis*	62
2.3	Metabolic substrates, enzymes, coenzymes and chemicals used in the study	68
3.1	The effect of culture age on the metabolic activity of mycoplasma cell suspensions in Ringer-HEPES buffer.	80
3.2	The effect of Ringer-HEPES buffer pH on metabolic activity of *M. bovis* type strain NCTC 10131.	80
3.3	Biochemical characteristics of *M. agalactiae* European strains). Relative rates of oxygen uptake.	82
3.4	Biochemical characteristics of *M. agalactiae* (European strains). K_s values	83
3.5	Biochemical characteristics of *M. bovis*. Relative rates of oxygen uptake.	84
3.6	Biochemical characteristics of *M. bovis*. K_s values	85
3.7	Biochemical characteristics of *M. bovigenitalium* and *M.* ovine serogroup11. Relative rates of oxygen uptake.	86
3.8	Biochemical characteristics of *M. bovigenitalium* and *M.* ovine serogroup11. K_s values.	87
3.9	Biochemical characteristics of *M. mycoides* SC. Initial rates of oxygen uptake.	99
3.10	Biochemical characteristics of *M. mycoides* SC. Relative rates of oxygen uptake.	99
3.11	Biochemical characteristics of *M. mycoides* SC. K_s values.	100
3.12	Biochemical characteristics of *M. mycoides* SC strain SH9 and *M. mycoides* LC	101
3.13	Change of pH during growth of *M. bovis* strain NCTC 10131 in different media.	105
3.14	Components of the modified PRM medium	114
4.1	NADH oxidase, GP oxidase activities and H_2O_2 production during NADH, GP oxidation by lysed cells of *M. agalactiae* (European strains).	135
4.2	NADH oxidase, GP oxidase activities and H_2O_2 production during NADH, GP oxidation by lysed cells of *M. bovis*.	136

4.3	NADH oxidase, GP oxidase activities and H_2O_2 production during NADH and GP oxidation by lysed cells of *M.* ovine serogroup 11 and *M. bovigenitalium*.	136
4.4	H_2O_2 production during oxidation of NADH and GP by whole cells of *M. agalactiae* (European strains), *M. bovis, M.* ovine serogroup11 and *M. mycoides* SC.	139
4.5	Production of H_2O_2 and oxygen uptake during oxidation of pyruvate and isopropanol by whole cells of *M. agalactiae*, *M. bovis*, *M.* ovine serogroup 11.	139
4.6	Effect of passage on H_2O_2 production by *M. bovis* strain 119B96. NADH oxidase activity and H_2O_2 production during oxidation of NADH by lysed cells of *M.bovis* 119B96 strain.	141
4.7	Substrate oxidation by zero, medium and high passage strain of *M. bovis* 119B96. Relative rates of oxygen uptake.	141
4.8	Glycerol kinase activity in *M. agalactiae, M. bovis*, and *M.* ovine serogroup 11 strains.	146
4.9	H_2O_2 production and NADH oxidation by lysed cells of *M. mycoides* SC.	147
5.1	Hydrolysis of SLPA-octanoate by *Mycoplasma* species	161
5.2	Hydrolysis of SLPA-hexanoate and SLPA-decanoate by *Mycoplasma* species	162
5.3	Hydrolysis of SLPA esters by *M. agalactiae* and *M. bovis* colonies	167
5.4	The effect of age on the hydrolysis of SLPA-octanoate.	168
5.5	Lipase activities in different fractions of *M. bovis* measured by spectrophotometric assay.	173
5.6	Lipase activities in different fractions of *M. bovis* measured by fluorimetric assay.	173
6.1	Identification of *M. bovis* strains by RFLP	183

List of figures

1.1	Phylogenetic tree inferred for some of the mycoplasmas belonging to the hominis group.	12
1.2	Mechanism of oxidative damage to host cells by adhering *M. pneumoniae*.	24
1.3	Catabolism of carbohydrate in *M. mycoides*	37
2.1	Clark-type oxygen electrode system	59
2.2	Structures of the novel chromogenic substrates used in the study	70
3.1	Calculation of K_s and V_{max} values for substrate utilisation from dissolved oxygen tension (DOT) versus time.	79

3.2	Rates of oxygen uptake by *M. bovis* and *M. agalactiae* type and field strains.	91
3.3	Rates of oxygen uptake by *M.* ovine serogroup 11 and *M. bovigenitalium* type and field strains.	91
3.4	Oxidation of pyruvate, 2 oxobutyrate, L-lactate and isopropanol by *M. agalactiae* NCTC 10123.	92
3.5	Oxidation of pyruvate, 2 oxobutyrate, L-lactate and isopropanol by *M. bovis* NCTC 10131.	92
3.6	Oxidation of pyruvate, 2 oxobutyrate, L-lactate and isopropanol by *M.* ovine serogroup 11.	93
3.7	Oxidation of pyruvate, 2 oxobutyrate, L-lactate and isopropanol by *M. bovigenitalium* 434/81.	93
3.8	The effect of organic acids and isopropanol on the growth of *M. bovis* type strain NCTC 10131.	106
3.9	The effect of organic acids and isopropanol on the growth of *M. bovis* 137B99.	106
3.10	The effect of organic acids and isopropanol on the growth of *M. agalactiae* type strain NCTC 10123.	107
3.11	The effect of organic acids and isopropanol on the growth of *M. agalactiae* strain 453/94.	107
3.12	The effect of organic acids and isopropanol on the growth of *M.* ovineserogroup 11 strain 2D.	108
3.13	The effect of organic acids and isopropanol on the growth of *M.* ovine serogroup11 strain 3SR99.	108
3.14	The effect of organic acids and isopropanol on the growth of *M.* ovine serogroup11 strain 48SR98.	109
3.15	The effect of organic acids and isopropanol on the growth of *M. bovigenitalium* type strain NCTC 10122.	109
3.16	The effect of organic acids and isopropanol on the growth of *M. bovigenitalium* strain 398/87.	110
3.17	Growth of *M. bovis* type strain NCTC 10131 in SP4 medium supplemented with pyruvate.	110
3.18	Growth of *M. bovis* type strain NCTC 10131 in PP medium supplemented with pyruvate.	111
3.19	Growth of *M. bovis* type strain NCTC 10131 in PRM medium supplemented with pyruvate.	111
3.20	Growth of *M. bovis* type strain NCTC 10131 in Eaton's medium supplemented with pyruvate.	112
3.21	Growth of *M. bovis* strain 82B96 in Eaton 's medium supplemented with increasing concentration of pyruvate.	112
3.22	Growth of *M. agalactiae* strain 101/94 in Eaton's media supplemented with increasing concentrations of pyruvate.	113
3.23	The effect of peptones (vegetable and non-vegetable source) on the growth of *M. bovis* strain 86B96.	113
3.24	Effect of the inhibitor α-methyl glucoside on the growth of *M. bovis* strain 79B96.	116

3.25	Effect of the inhibitors citrulline, lysine and ornithine on the growth of *M. bovis* strain 79B96.	116
3.26	Effect of the inhibitor penicillin on the growth of *M. bovis* strain 79B96.	117
3.27	Effect of the inhibitor α-methyl glucoside on the growth of *M.* ovine serogroup 11 strain 2D.	117
3.28	Effect of the inhibitors citrulline, lysine and ornithine on the growth of *M.* ovine serogroup 11 strain 2D.	118
3.29	Effect of the inhibitor penicillin on the growth of *M.* ovine serogroup 11 strain 2D.	118
3.30	Effect of the inhibitor α-methyl glucoside on the growth of *M.* ovine serogroup 11 strain 95SR99.	119
3.31	Effect of the inhibitors citrulline, lysine and ornithine on the growth of *M.* ovine serogroup 11 strain 95SR99.	119
3.32	Effect of inhibitor penicillin on the growth of *M.* ovine serogroup 11 strain 95SR99.	120
4.1	Glycerol oxidation pathway in *M. mycoides*	124
4.2	H_2O_2 production during NADH oxidation by *M. agalactiae* NCTC 10123 (A) and1070/93 strain (B).	127
4.3	H_2O_2 production during NADH oxidation by *M. bovis* NCTC 10131(A) and 81B96 strain (B).	128
4.4	H_2O_2 production during oxidation of organic acids by *M. agalactiae, M. bovis, M. bovigenitalium* and *M.* ovine serogroup 11	126
4.5	Production of H_2O_2 during NADH oxidation by *M.* ovine serogroup 11, 2D (A) and 50SR98 strain (B).	129
4.6	H_2O_2 production during NADH oxidation by *M. bovigenitalium* 434/81(A) and 398/87 strains (B).	130
4.7	Production of H_2O_2 during NADH oxidation by *M. bovis* 119B96 low passage (A)119B96 passage 50 strain (B) 119B96 passage 200 strain (C).	142
5.1	The catalytic action of lipases.	151
5.2	Hydrolysis of SLPA-octanoate by colonies of *M. agalactiae.*	154
5.3	Effect of pH on lipase activity of *M. bovis* cells in phosphate buffer (A) and Ringer-HEPES (B).	169
5.4	Effect of different temperatures on lipase activity in *M. bovis* cells.	170
5.5	Effect of detergents on lipase activity in *M. bovis* cells.	170
5.6	Detection of lipase activity in *M. bovis* on native PAGE (12.5% w/v).	174
6.1	The quantification of *M. bovis* DNA by 0.7 % (w/v) agarose gel electrophoresis.	179
6.2	The confirmation of *M. bovis* strains by PCR.	179

6.3	RFLP of eighteen *M. bovis* strains using restriction enzyme *Asn*I.	180
6.4	RFLP of eighteen *M. bovis* strains using restriction enzyme *Ssp*I.	181
6.5	RFLP of eighteen *M. bovis* strains using restriction enzyme *Dde*I.	182
6.6	Cleavage patterns of the DNA of *M. bovis* strains by *EcoR* I.	185
6.7	Cleavage patterns of the DNA of *M. bovis* strains by *Hind*III.	185
6.8	Cleavage patterns of the DNA of *M. bovis* strains by *Hind* III, *Bgl* I, *Bam*HI, *Pst*I, *Sma*I, and *EcoR*I.	186
6.9	PFGE of *Sma*I digest of genomic DNA of *M. bovis* strains.	186
6.10 a	SDS-PAGE (12.5% w/v) pattern of whole cell protein from *M. bovis* strains.	190
6.10 b	SDS-PAGE (12.5 % w/v) pattern of whole cell protein from *M. bovis* strains.	190
6.10c	SDS-PAGE (12.5 % w/v) pattern of whole cell protein from *M. bovis* strains.	191
6.11	SDS-PAGE (12.5 % w/v) pattern of whole cell protein from *M. bovis* 119B96 passage strains.	191
6.12	SDS-PAGE (12.5 % w/v) pattern of whole cell protein from *M. agalactiae* strains.	193
6.13	SDS-PAGE (12.5 % w/v) analysis of *M. agalactiae* and *M. bovis* strains whole cell protein.	193
6.14	SDS-PAGE (12.5 % w/v) analysis of *M. bovis* and *M.* ovine serogroup 11, and *M. bovigenitalium* strains whole cell protein.	194
6.15	Immunoblots of whole cell proteins of *M. bovis* strains.	198
Appendix 1 figures	Representative standard curve of bovine serum albumin (BSA) at 660 nm Markwell method (1978)	251
	Representative standard curve of bovine serum albumin (BSA) Bradford method (1976).	251
Appendix 2 figures	Representative standard curve of SLPA-phenol	252
	Representative standard curve of 4-methylumbelliferone	252
	Absorption spectra of the SLPA-phenol 0.2 mM using scanning spectrophotometer (PU 8720 UV/VIS, Philips)	253

List of abbreviations

ADH	Alcohol dehydrogenase
AIDS	Acquired immunodeficiency syndrome
BSA	Bovine serum albumin
CBPP	Contagious bovine pleuropneumonia
CCPP	Contagious caprine pleuropneumonia
Cfu	Colony forming unit
DMSO	Dimethyl sulphoxide
DOT	Dissolved oxygen tension
EDTA	Ethylenediaminetetraacetic acid
EDH	Ethanol dehydrogenase
ELISA	Enzyme linked immunosorbent assay
EMP	Embden Meyerhof-Parnas pathway
FAD	Flavin adenine nucleotide
G+C	Guanine plus cytosine
GlcNAc	N-acetyl glucosamine
GP	L-α-glycerophosphate
HEPES	N-(2-Hydroxyethyl) piperazine-N'-2-ethanesulfonic acid
HIV	Human immunodeficiency virus
HMS	Hexose monphosphate shunt
H_2O_2	Hydrogen peroxide
IL	Interleukin
KDa	Kilodalton
MLOs	Mycoplasma like organisms
4-MU	4-Methylumbelliferone
NAD^+	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide, reduced form
$NADP^+$	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced form
NER	Nucleotide excision repair
O_2^-	Superoxide anion
$OH^\bullet$	Hydroxyl ion
ORFs	Open reading frames
PCR	Polymerase chain reaction
PEP:PTS	Phosphoenolpyruvate: phosphotransferase system
PFGE	Pulsed field gel electrophoresis
PFK	Phospho fructokinase
RFLP	Restricted fragment length polymorphism
ROS	Reactive oxygen species
RH	Ringer-HEPES solution
SC	Small colony
SLPA	Syringaldehyde lepidine propionic acid .
SOD	Superoxide dismutase , SD Standard deviation
TCA	Tricarboxylic acid
TNF-α	Tumor necrosis factor alpha
Tris	Tris (hydroxymethyl) aminomethane
VSP	variable surface protein

Chapter 1

1 Introduction

1.1 Mollicutes

Mollicutes are prokaryotic organisms characterised by the absence of a cell wall; they have no peptidoglycan and consequently are resistant to antibiotics such as penicillin, cycloserine and others, which inhibit peptidoglycan biosynthesis. Members of the class *Mollicutes* are eubacteria that are bounded by a single trilaminar membrane. Because they lack a cell wall and cannot synthesise all wall precursors such as muramic and diaminopimelic acid (Plackett, 1959; Martin *et al.*, 1980) they are osmotically fragile and exhibit plasticity and pleomorphism. They vary in shape from spherical or pear-shaped structures to unbranched, branched or helical filaments and can pass through membrane filters of 220-450 nm. Most of the mollicutes are facultatively anaerobic and some are obligate anaerobes. They grow well in both anaerobic and aerobic environments at a pH of 7.6-7.8 and usually grow well in sealed liquid broth cultures. Gentle vigorous aeration increases the growth rate and yield of some such as *M. mycoides* subsp. *mycoides.* Mycoplasma colonies have a tendency to grow into the medium (Rodwell and Mitchell, 1979; Razin and Freundt, 1984) giving most of them are a typically "fried egg" appearance

The organisms can be very small; the diameter of some viable spherical cells is in the range of 300 nm (Boatman, 1979) and viable helical filaments can be as small as 20 nm in diameter (Whitecomb and Tully, 1984). Mollicutes are the pathogens or commensal organisms of a wide range of human, animals, plants and insects. All known mycoplasmas are parasites of various animal hosts; and several species cause respiratory, arthritic, and urogenital diseases associated with severe morbidity and mortality in vertebrate animals (Razin *et al.*, 1998) including humans (Taylor-Robinson and Bradbury, 1998). Mycoplasmas are the cause of some progressive chronic diseases and are difficult to control due to their innate resistance to murein synthesis inhibitors since they are devoid of a cell wall (Razin *et al.*, 1998); the development of resistance to other antibiotics through mutations or the acquisition of mobile genetic elements is increasingly seen (Taylor-Robinson and Bebear, 1997).

Due to their small size, mollicutes have restricted metabolic activities and catabolism is primarily associated with ATP generation rather than the synthesis of metabolic precursors for anabolic metabolism (Miles, 1992b). However Manolukes *et al.* (1988) demonstrated potential pathways, whereby products of carbohydrate metabolism in *Mycoplasma* and *Acholeplasma* may be used in the synthesis of lipids, nucleic acids and certain amino acids. Mollicutes are important pathogens and increased knowledge of their structure, physiology and metabolism may help in understanding their pathogenesis and the development of control and treatment strategies.

1.2 Classification of Mollicutes

The absence of cell wall, the typical "fried egg" colonies and the requirement for exogenous sterol are the most important criteria for the classification of mollicutes from other prokaryotes (Razin and Freundt, 1984). Among mollicutes, *Mycoplasma*, *Ureaplasma, Entomoplasma, Spiroplasma* and *Anaeroplasma* require sterol but the exceptions of *Acholeplasma, Asteroplasma* and *Mesoplasma* genera do not require sterol for their growth. The families and genera of mollicutes are distinguished on the basis of a small number of structural, nutritional and other features (Table 1.1). Mollicutes are presently the only cell wall-less class in the division *Tenericutes*, one of the four divisions of the kingdom (Murray, 1984). The other three divisions are *Firmicutes* (the gram-positive bacteria), *Gracilicutes* (the gram negative bacteria) and *Mendosicutes* (the archaebacteria). The ability of some species to accumulate and catabolise arginine via the arginine dihydrolase pathway is used to separate mycoplasma into glycolytic and arginine utilising species (Barile *et al.*, 1996). Another important factor is that these organisms are the smallest known cells, with a small genome and have a G + C content of 23-41 mol %, which is at the low end of the range of values found in bacteria (Maniloff *et al.*, 1992).

The class *Mollicutes* has four families: *Mycoplasmataceae, Acholeplasmataceae, Asteroplasmataceae, and Spiroplasmataceae.* Members of the class *Mollicutes* have been shown to have only one or two copies of rRNA genes, compared with eight to ten copies in most bacteria (Razin, 1985). Acholeplasmas and phytoplasmas have two rRNA operons, while the spiroplasmas and mycoplasmas have either one or two copies (Amikan *et al.*, 1984; Schneider and Seemuller, 1994). It has recently been shown by 16S rRNA sequence

analysis that the haemotrophic bacteria *Eperythrozoom* sp. and *Haemobartonella* sp. which were earlier classified as rickettsias, are closely related to the mollicutes (Neimark and Kocan, 1997; Rikihisa *et al.*, 1997). These haemotrophic bacteria also lack a cell wall and they cluster with the mollicutes of the pneumoniae group.

Identification and classification of members of the class *Mollicutes* have been based on phenotypic characteristics, such as the ability to produce acid from glucose, requirement of urea for growth, and utilisation or hydrolysis of arginine, on serological associations, and on rRNA sequence. The members of the class *Mollicutes* have undergone a remarkable series of genome reductions, as determined by rRNA analysis. Their genome consists of only 600 kb to 1500 kbp which makes them highly dependent upon their host for survival (Baseman and Tully, 1997). As a result, this class of organisms contains some of the smallest genomes presently known among free-living organisms (1100 kbp, *E. coli;* Myers *et al.*, 1980). The genome of four *Mycoplasma* species; *M. genitalium* (580 kbp); *M. pneumoniae* (816 kbp); *U. urealyticum* (752 kbp) and *M. pulmonis* (964 kbp) have been sequenced completely (Fraser *et al.*, 1995; Himmelreich *et al*; 1996; Glass *et al.*, 2000; Chambaud *et al.*, 2001).

The 16S rRNA genes of mycoplasmas, have been the subjects of study mainly for the purpose of taxonomy. The degree of similarity between 16S rRNA sequences were interpreted as evolutionary relatedness and the mollicutes were divided to 5 phylogenetic groups. The groups, hominis, pneumoniae, spiroplasmas, anaeroplasmas and asteroplasmas were further divided into 16 clusters (Weisberg *et al.*, 1989).

1.3 Genus *Mycoplasma*

Mycoplasmas are a large group of diverse prokaryotic species. They consist of the smallest known self-replicating organisms phylogenetically related to gram-positive eubacteria in the *Bacillus Lactobacillus-Streptococcus* subgroup of gram +ve bacteria on the basis of 16S rRNA gene sequence analysis. They are related even more so to a particular small subgroup of clostridia represented by *Clostridium innocum* and *Clostridium ramosum* (Woese *et al.*, 1980).

Table 1.1 General characteristics and taxonomy of the class *Mollicutes* (Razin *et al*, 1998, modified).

Classification	Genome size (kbp)	G C contents of DNA (mol %)	Sterol requirement	Distinctive features	Hosts	Diseases / organ affected
Order I: *Mycoplasmatales* **FamilyI:** *Mycoplasmataceae* **Genus I:** *Mycoplasma*	580-1350	23-40	yes	Optimum growth at 37 °C Pleomorphic, spherical, ovoid or pear shaped and/or slender branched filaments. Gram-ve, non-motile but gliding motility is found. Facultatively anaerobes and catalase negative.	Animals, man, plants and insects	Respiratory, urogenital , joint fluids, oral cavity and udder
Genus II: *Ureaplasma*	760-1170	27-30	yes	Urea hydrolysis, pleomorphic, coccobacillary, non-motile and gram-ve. Arginine hydrolysis and other carbohydrate are not metabolised. Colonies are 15-60 μm in diameter.	Man and animals	Urogenital tract,oral, respiratory and arthritic. Associated with non-gonococcal arthritis, stillbirth spontaneous abortion, premature and low weight birth (Waites *et al.*, 1990; Cassell *et al.*, 1993). Cause reduced human sperm motility (Rose and Scott, 1994).

Continued

Classification	Genome Size (kbp)	G C contents of DNA (mol %)	Sterol Req.	Distinctive features	Hosts	Diseases / organ affected
Order II: *Entomoplasmatales* **Family I:** *Entomoplasmataceae* **Genus I:** *Entomoplasma*	790-1140	27-29	yes	Optimum growth at 30°C. Non-helical mollicutes (Tully *et al.*, 1993)	Plants and insects	Not known
Genus II: *Mesoplasma*	870-1100	27-30	no	Optimum growth at 30°C. Non - helical mollicutes	Plants and insects	Not known
Family II: *Spiroplasmataceae* **Genus I:** *Spiroplasma*	780-2220	24-31	yes	Helical filaments, growth at 30-37°C. Pleomorphic, branched and non-helical filaments, motile. Facultatively anaerobes. Colonies are diffused and satellite, of size 0.1-4 mm and most strains hydrolyse arginine. No hydrolysis of urea, arbutin or esculin. They possess spirilin protein associated with motility. Isolated from rabbit ticks, haemolymph and guts of insects and fluids of plants.	Arthro-pods and plants	Isolated from phloem and surfaces of plants. Corn stunt citrus stubborn. Lethargy of bettles and insects. *Spiroplasma. citri* and *S. kunkelii* cause citrus stubborn and corn stunt. *S. micum* causes suckling mouse cataract agent. Also cause of brain infection and damage in suckling mouse (Whitcomb and Williamson, 1975). Spiral-like inclusions demonstrated from Creutzfeldt-Jacob disease (Bastian *et al.*, 1981). Mycoplasma-like organisms cause yellow stunting shoot. Proliferation and phyllody (McCoy *et al.*, 1989). *S. melliferum* honey bee spiroplasmosis (Clark *et al.*, 1985).

Continued

Classification	Genome size (kbp)	G C contents of DNA (mol %)	Sterol req	Distinctive features	Hosts	Diseases / organ affected
Order III: *Acholeplasmatales* **Family I:** *Acholeplasmataceae* **Genus I:** *Acholeplasma*	1500-1650	26-36	no	Optimum growth at 30-37°C. Colony size 2-3 mm in diameter and fried egg shape. Arginine and urea are not hydrolysed. Carbohydrates are fundamental substrates. NADH is located in the plasma membrane. Agar colonies show haemadsorption of RBC. Gram–ve, non-motile and facultative anaerobes. Spherical and filaments	Animals plants and insects	Respiratory, arthritic and urogenital diseases. Commensal of nasal, oral cavity and intestinal tract. *A. axanthum* cause lethal yellowing disease of coconut tissue (Eden and Tully, 1979). *A. oculi* associated with vaginitis and cervicitis of goats. Also isolated from soil (Laidlaw and Elford, 1936).
Order IV: *Aneroplasmatales* **FamilyI:** *Aneroplasmataceae* **GenusI:** *Anaeroplasma*	1500-1600	26-34	yes	Obligate anaerobes. Coccoid, Pleomorphic, gram-ve, non-motile, fried egg colonies, and are metabolically quite distinct	Bovine, ovine rumen	Commensal rumen flora
Genus II: *Asteroplasma*	1500	40	no	Obligate anaerobes	Bovine and insects	Commensal rumen flora
Undefined taxonomic status phytoplasmas	649-1185	23-29	Not known	Uncultured *in vitro*	Plants and insects	Cause diseases in plant species (McCoy *et al.*, 1989). Cause abnormal yellowing of leaves, stunted growth and premature flowering in *P. tenax.* Cause degeneration of vascular bundles, root death and rhizome rooting (Boyce *et al.*, 1951).

Despite their very small genomes, mycoplasmas are successful pathogens of man and a wide range of animal hosts such as fish (Kirchhoff and Rosengarten, 1984) and sea mammals (Giebel *et al.*, 1991). Recently, new species have been isolated from the desert tortoise, *Mycoplasma agassizii* (Brown *et al.*, 2001b) and *Mycoplasma alligatoris* from American alligators (Brown *et al.*, 2001a). The pathogenic mycoplasmas usually parasitise the mucosal surfaces of the respiratory and urogenital tract, rarely penetrating the submucosa or blood (Maniloff *et al.*, 1992). *Mycoplasma* species are usually restricted to a single host species, although some have a wide host range; for example *M. arginini* has been isolated from sheep, goats and pigs (Kazama *et al.*, 1994). Some species may also survive as contaminants of animals cell cultures such as *Mycoplasma genitalium* (McGarrity *et al.*, 1992).

Mycoplasmas are recognised primarily as extracelluar parasites or pathogens of mucosal surfaces although certain species may invade host cells (Taylor-Robinson *et al.*, 1991). They are also linked with other infectious agents including HIV (Bauer *et al.*, 1991), meningiococci (Hierholzer *et al.*, 1991) and chlamydia (Sherman *et al.*, 1990). The ability of mycoplasmas to induce biologically significant processes in cell culture is of great interest as it has been estimated that from 10 to 80 % of all tissue cultures are contaminated by mycoplasmas (Razin *et al.*, 1998). Several species of *Mycoplasma* are well-established pathogens causing diseases in man and animals (Simecka *et al.*, 1992; Table 1.2).

The first mycoplasma isolated was the causal agent of contagious bovine pleuropneumonia (CBPP), *Mycoplasma mycoides* subsp. *mycoides* SC (Nocard and Roux, 1898). It is the only bacterial disease included in the A list of communicable animal diseases of the Office International des Epizooties (OIE) and is the most important animal disease in Africa, affecting at least 27 countries (Nicholas *et al.*, 2000). CBPP was eradicated from most of Europe at the end of the 19th century but reappeared sporadically and affected several countries in an epizootic in Southern Europe between 1983 and 1999 with outbreaks of CBPP in France, Portugal, Spain and Italy (Ter Laak, 1992). *M. mycoides* subsp. *mycoides* SC belongs to the *M. mycoides* cluster, a group of closely related infectious mycoplasmas including *M. mycoides* subsp. *mycoides* large colony (LC), *M. mycoides* subsp. *capri, Mycoplasma capricolum* subsp. *capricolum, M. capricolum* subsp. *capripneumoniae* and *Mycoplasma* sp. bovine group 7, (Cottew *et al.*, 1987).

Table 1.2 Mycoplasma diseases of animals (Simecka *et al.*, 1992, modified).

Mycoplasma	host	disease	Mycoplasma	host	disease
M. arthritidis	Rats	Arthritis, murine respiratory,	*Mycoplasma* sp.		Mastitis
M. pulmonis	and mice	genital and arthritis	strain GM 790 A		
M. alkalescens	cattle	Mastitis and genital diseases	*M. capricolum*	Goats	Respiratory diseases and arthritis
M. bovirhinis		Mastitis	*M. agalactiae*		Mastitis and arthritis
M. bovis		Calf pneumonia, mastitis, and arthritis	*M. mycoides* ssp.	Sheep and goats	Pleuropneumonia and arthritis
M. bovoculi			*mycoides* LC		
M.californicum		Mastitis	*M. ovipneumoniae*		Proliferative interstitial pneumonia.
M. canadense			*Mycoplasma* sp.	Sheep	Pneumonia
M. dispar		Contagious bovine pleuropneumonia, arthritis	Strain F.38		Pleuropneumonia similar to classic
M. mycoides subsp. *mycoides* SC			*M. conjunctivae*		Pleuropneumonia
			M. hyorhinis	Swine	Conjunctivitis
Mycoplasma sp. group 7		Calf pneumonia and arthritis	*M. hyosynoviae*		Enzootic pneumonia
			A. axanthum		
Ureaplasma sp.		Calf pneumonia and arthritis	*M. gallisepticum*	Poultry	Pneumonia and arthritis
M. putrefaciens	Goats		*M. iowae*		Infectious air saculitis (turkeys), and sinusitis (chicken), mild air sacculitis, air saculitis and arthritis (chicken and turkey)
		Conjunctivitis	*M. meleagridis*		
			M. synoviae		

1.3.1 *Mycoplasma agalactiae* and *Mycoplasma bovis*

M. agalactiae was first isolated and cultured in 1925 and was the second mycoplasma to be discovered. *M. agalactiae* is the major etiological agent of contagious agalactia, a disease of small ruminants of considerable economic importance in Mediterranean countries; it has been isolated worldwide (Cottew, 1979; DaMassa *et al.,* 1992). No continent appears to be free of the disease causing agalactia, mastitis, arthritis and keratoconjuctivitis (Lambert, 1987). *Mycoplasma mycoides* subsp. *mycoides* LC, *Mycoplasma mycoides* subsp. *capri, Mycoplasma capricolum* subsp. *capricolum* may induce similar diseases, often associated with clinical signs of respiratory distress. In areas where the infection is endemic, only asymptomatic or sub-clinical mastitis can be observed, and are rarely associated with clinical signs of keratoconjutivitis or arthritis (Gourlay, 1981).

M. agalactiae can remain dormant for many months in ewes until conditions such as lactation occur favouring their development, after which the disease breaks out (Cottew, 1979). This mycoplasma is phylogenetically closely related to *M. bovis* (Mattsson *et al.*, 1994), an important bovine pathogen causing mastitis, arthritis and respiratory disease in cattle (Boughton, 1979; Ter Laak *et al.,* 1992). The close relationship between the two species is manifested by the high degree of homology of the 16S rRNA genes of both species (Mattsson *et al.*, 1994) and by the presence of shared antigens detected with polyclonal or monoclonal antibodies (Askaa and Ernø, 1976; Rasberry and Rosenbusch, 1995). On the other hand DNA-DNA hybridisation experiments have shown only 40 % homology between *M. agalactiae* and *M. bovis* genomes, a value which clearly indicates that these two species are two distinct species (Askaa and Ernø, 1976). *M. agalactiae* is reported to have a mol % of G + C in the range of 33.5-34.2 (Freundt and Edward, 1979).

M. bovis is almost always associated with other pathogens such as *Haemophilis somnus, Pasteurella multocida. M. bovis* was first isolated from severe bovine mastitis in 1961 in the US (Hale *et al.*, 1962) and initially called *M. agalactiae* var *bovis* but was elevated to a separate species in 1976 (Asakaa and Ernø, 1976). It was first isolated in Britain from a severe case of calf pneumonia in the south of England (Reilly *et al.,* 1993). Continuous or intermittent shedding of *M. bovis* by pneumonic or mastitic animals is regarded as the most important source of infection, especially during the milking operation and herd expansion

programmes (Pfutzner and Sachse, 1996). While *M. bovis* was found to be most closely related to *M. agalactiae,* it is in a distinct cluster together with other non-fermenting, non-arginine hydrolysing mycoplasmas *M. lipophilum,* *M. bovigenitalium* and *M. californicum* (Mattsson *et al.*, 1994; Figure 1.1). The branching order of the other species is in agreement with earlier data on mycoplasma phylogeny (Weisberg *et al.*, 1989). *M. bovis* diseases occur throughout the world leading to extensive economic losses for both dairy and meat production (Nicolet, 1994). Its incidence is growing in Europe with the increase of the beef trade (Ter Laak *et al.*, 1992). *M. bovis* strain 1067 isolated from a case of bovine mastitis (Poumarat *et al.*, 1985) was used successfully to experimentally induce reproducible arthritis in calves (Belli *et al.*, 1989). *M. bovis* is considered to be one of the most pathogenic *Mycoplasma* species in cattle. It is a well-known established aetiologic agent of mastitis (Brown *et al.*, 1990), arthritis (Pfutzer, 1984), and pneumonia (Gourlay *et al.*, 1989) and has also been reported to cause diseases of the genital tract (Ruhnke, 1994), abscesses (Kinde *et al.*, 1993) and meningitis (Stipkovits *et al.*, 1993).

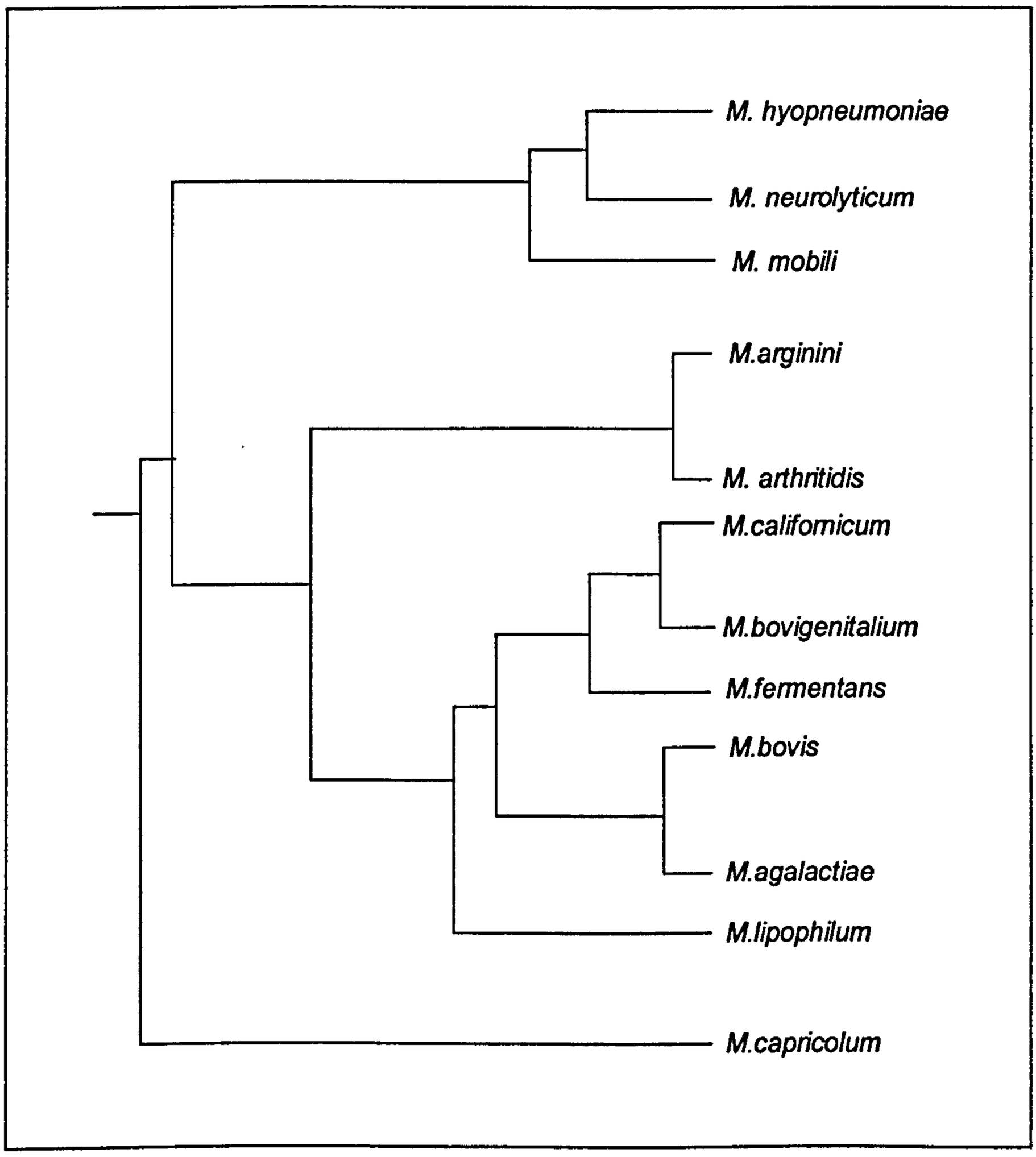
Furthermore, intrauterine application produced abortions in cows and intravesicular application led to vasculitis, epididymitis and orchitis in bulls (Bocklisch *et al.*, 1986). *M. bovis* has been isolated from the tympanic bullae of dairy calves with an exudatative otitis media (Walz *et al.*, 1997). It has been estimated to cost the US cattle industry at least 32 million dollars in mortality and set-back losses annually (Rosengarten and Citti, 1999). In Britain and France only, *M. bovis* is believed to be responsible for 25-33 % of outbreaks of calf pneumonia (Nicholas *et al.*, 2000). *M. bovis* and *M. mycoides* subsp. *mycoides* are considered to be the most pathogenic species for cattle (Gourlay and Howard, 1979). The mechanism by which *M. bovis* causes damage is thought to be principally through attachment to host cells (Thomas *et al.*, 1991), and cell injury as a result of production of toxic substances, such as a complex polysaccharide (Geary *et al.*, 1981). It has been associated with up to 52.2 % of mycoplasma mastitis in California as reported by Jasper (1982), and according to Gourlay *et al.* (1989) up to 36.5 % of mortality of calves due to respiratory tract disease in the UK. In recent years, the spread of this pathogen in cattle has been rapid and extensive (Nicolet, 1994). Recently *M. bovis* was reported to be associated with apoptotic death of bovine lymphocytes (Tony *et al.*, 2002). It has also been shown to induce bovine macrophages to produce TNF-α and nitric oxide, two powerful initiators of

immune activity (Jungi *et al.*, 1996). Due to their resistance to antibiotic therapy and the lack of commercially available sensitive diagnostic tools and effective vaccines. *M. bovis* induced diseases are difficult to prevent and control. *M. bovis* infections are chronic in nature. Immune invasion mechanisms, which enable them to rapidly change the structure and expression of some of their membranes, surface proteins exposed to the host immune system (Wise *et al.*, 1992). It is often suggested that *M. bovis* cannot persist in the environment outside the host animal for a longer period because of high susceptibility to dryness, however in deep frozen semen processed for artificial insemination, the agent remained infective for years Pfutzner (1984). Nagatomo *et al.* (2001) recently reported that *M. bovis* persisted for 59-185 days in liquid medium regardless of the medium components or temperature.

M. bovis has also been isolated from goats (Chima *et al.*, 1986), horses, swine (Kumar *et al.*, 1987), geese, ducks (Gailaard *et al.*, 1983) and man (Madoff *et al.*, 1979). Sheep can acquire *M. bovis* from infected cattle and should also be taken into account as a potential vectors (Bocklisch *et al.*, 1986). *M. bovis* has been isolated from the stomach contents of an aborted foetus in Ireland (Byrne *et al.*, 2001).

M. bovis and *M. agalactiae* are closely related organisms showing only eight nucleotide differences in evolutionary variable regions of the 16S rRNA gene (rrs) resulting in 99% nucleotide similarity (Mattsson *et al.*, 1994). Ribosomal RNA sequences can be used as powerful molecular chronometers and have been employed to determine the phylogenetic interrelationships of microorganisms (Olsen *et al.*, 1993). The 16S rRNA sequence from *M. bovis* was partially determined by a reverse transcription sequencing method adapted for rRNA (Mattsson *et al.*, 1991). Genetic methods such as using oligonucleotides complementary to variable regions of the rrs gene (Mattsson *et al.*, 1991) and polymerase chain reaction amplification of the 16S rRNA gene (Gonzalez *et al.*, 1995) and PCR based on randomly cloned DNA fragments have been used to detect *M. bovis* (Tola *et al.*, 1996). *M. bovis* has been detected in milk samples by PCR (Hotzel *et al.*, 1999). Recently *M. bovis* was characterised with arbitrarily primed polymerase chain reaction (AP-PCR) (Butler *et al.*, 2000). Genetic and antigenic variation within the *M. bovis* type strain and field isolates have been found (Behrens *et al.*, 1994), however Poumarat *et al.* (1994) have reported that antigenic variations might interfere with these identification procedures.

Figure 1.1 Phylogenetic tree inferred for some of the mycoplasmas belonging to the hominis group. The tree was inferred from 16S rRNA sequence data with *M. capricolum* as an out group (Mattsson *et al.*, 1994)



Therefore a strategy based on stable genes, which are not involved in genetic variability, may be used. Housekeeping genes, which are expressed in all living organisms, are good candidates for the genetic differentiation of species. The *uvr*C gene encodes deoxyribodipyrimidine photolyase (EC 4.1.99.3) and is an enzyme of the excision DNA repair system, *uvr* ABC, which removes damaged DNA segments via concerted dual incisions by ATP-dependent enzyme systems with an essentially infinite substrate range (Sancar *et al.*, 1984). This system has been found in all free-living species from the smallest self-replicating organism like *M. genitalium* to humans (Sancar, 1994). *Uvr*D has been implicated in DNA replication and recombination (George *et al.*, 1994). The *uvr*C gene sequences had been used to design the specific primers for the identification of *M. agalactiae* and *M. bovis* (Subramaniam *et al.*, 1998).

1.4 Morphology and cell division

Mycoplasma cells are bound by a cell membrane only; their dominating shape is a sphere. However many mollicutes exhibit a variety of morphological entities, including pear-shaped, flask-shaped with terminal tip structures, filaments of various lengths, and helical filaments. Shimizu and Miyata, (2002) have recently reported flask-like cell morphology of *M. mobile* by electron microscopy. In most cases, elongated fusiform, bottle shaped can be observed, the dominant shape being dependent on the *Mycoplasma* species and on growth conditions. The ability to maintain such shapes in the absence of a rigid cell wall has long indicated the presence of a cytoskeleton in mycoplasmas (Razin, 1978).

In spiroplasma, the filaments are helically coiled 0.08-0.2 μm thick and usually 2-5 μm long. Mycoplasma filaments are usually 0.2-0.4 μm and may reach a length of over 100 μm and show true branching. The diameter of the coccoid bodies usually varies between 0.3-0.8 μm and is considered to be the smallest cells capable of reproduction (Razin, 1978). Filaments growth is usually transitory, as filaments transform into chains of cocci that later break apart (Razin and Cosenza, 1966; Bredt *et al.*, 1973).

Helical morphology, rotatory and undulating motility of helical filaments are the major criteria by which the family *Spiroplasmataceae* differ from other genera (Cole *et al.*, 1973). Cinematography of single cells showed division occurring by binary fission e.g. in

M. gallisepticum and fragmentation of filaments rings by a budding process (Morowitiz and Maniloff, 1966).

Anderson and Barlie (1965) suggested that mycoplasma cells have several alternative modes of reproduction, depending on cultural conditions. Mycoplasmas do not have any appendages such as flagella or pili (Kirchhoff, 1992) or any genes obviously related to motility, including those for motor proteins such as myosin or kinesin (Fraser *et al.*, 1995; Himmelreich *et al.*, 1996; Chambaud *et al.*, 2001). *M. pneumoniae* cells have a simple structure which is characterised by spindle shaped cells with a distinct asymmetry due to the presence of a polar terminal structure; the attachment organelle and its bifurcation is thought to be an early step in cell division (Boatman, 1979; Seto *et al.*, 2001). This terminal structure is the leading edge in gliding motility, and its duplication is thought to precede cell division, raising the possibility that mutations affecting cytadherence are also involved in motility or cell development (Cynthia *et al.*, 1999). Gliding motility, a movement by which cells attach to various matrices and slide on them, has been reported for several *Mycoplasma* species, including *M. pneumoniae*, *M. pulmonis*, *M. gallisepticum*, *M. genitalium* and *M. mobile* (Kirchhoff, 1992). Miyata *et al.* (2000) reported that gliding motility plays a critical role in microcolony formation, suggesting that gliding mycoplasmas may use their motility not only for migration to the preferred locations in the host, but also for successful colonisation of host tissues.

Haemadsorption activity correlates to the binding activity required for gliding, suggesting that the machinery for cell adhesion is also used for gliding motility. About 15 genes and corresponding proteins are responsible for the division process in *E. coli*, but only three genes (*fts*H, Y, Z) of cell division group were found in the completely sequenced genomes of *M. genitalium* and *M. pneumoniae* (Fraser *et al.*, 1995, Himmelreich *et al.*, 1996). However, cell division protein *fts*Z of *Ureaplsama urealyticum* appears not to be encoded (Glass *et al.*, 2000). The functions of *fts*H and *fts*Y gene products in bacterial proliferation are uncertain whereas *fts*Z plays a pivotal role in cell division. In bacterial cells, *fts*Z forms a scaffold of contractile rings and is located at the leading edge of the invaginating septum during the division process (Lutkenhaus and Addinall, 1997).

Mycoplasma cell morphology can be influenced by a variety of factors; for example, supplementation with unsaturated fatty acids induces filament formation in *M. gallisepticum* (Rodman and Mitchell, 1979). Spiroplasmas are typically helical filaments in culture but are coccoid inside host cells, perhaps due to changes in ion concentrations (Hackett and Clark, 1989). In *M. mycoides*, morphology is dependent upon the degree of saturation and chain length of fatty acids (Rodwell and Peterson, 1971). Morphology is also affected by the pH and osmotic pressure of the growth medium and the specific growth rate (Henderson and Miles, 1990).

1.5 Colony shape

Colonies on solid medium are usually smaller than 1 mm in diameter and the organism tends to penetrate and grow beneath the surface of solid media under suitable conditions, however colonies of *M. bovis*, *M. mycoides* and *M. bovoculi* are large and may exceed 2 mm in diameter. Most non-motile species form colonies that have characteristics of "fried egg" and retain Diene's stain. Colonial appearance depends to a great extent upon the composition of growth medium, which may determine the size of the colonies, the abilities of their central areas to grow down into agar medium, and the extent to which the peripheral surface growth develops (Razin, 1983). Larger classical "fried egg" colonies may develop on optimal media (Shphared *et al.*, 1974). The colonial appearance may also be modified by phage infection (Mel nikove and Klisunova, 1972).

1.6 Ultrastructure

Mycoplasmas exhibit extremely simple ultrastructure. The mycoplasma cell is bound by a plasma membrane (8.5–10 nm) and the enclosed cytoplasm contains ribosomal circular double DNA molecule and no intracellular membrane structures. Mycoplasma membrane resembles plasma membrane of other prokaryotes in being composed of two-thirds proteins and one-third lipids. Membrane proteins, glycolipids and lipoglycan exposed on the cell surface are major antigenic determinants in mycoplasmas.

M. pneumoniae has been demonstrated to be filamentous and to attach to the luminal surface of respiratory epithelium by a specialised tip structure (Collier and Clyde, 1974). In

M. mycoides the capsule consists of 10 % of the dry weight of the cell (Howard and Gourrlay, 1974) and is composed of approximately 90 % galactose and 10 % glucose (Plackett and Buttery, 1958), while *M. mycoides* subsp. *capri* consists largely of glucose with a small amount of galactose (Schiefer *et al.*, 1974).

1.7 Membrane structure

The plasma membrane of mollicutes contains 50-60 % proteins and 30-40 % lipids (Razin, 1979). Carbohydrates present as either polysaccharides or lipopolysachrides and may account for as much as 10-15 % of the membrane dry weight in *Acholeplasma laidlawii* and in some mycoplasmas (Kahane and Schiefer, 1983). The lipids of the mollicutes are located exclusively in the cytoplasmic membrane, constituting 25-35 % of the dry weight (Smith, 1992). Since cells possess only a single cell membrane, the membrane contains essentially all of the cellular lipid and, a substantial fraction of the total cellular protein as well. Large variations in lipid/protein ratios can be induced by variation in culture conditions, particularly by changes in the type and amount of exogenous fatty acid added to the growth medium. This is because of the mollicutes limited abilities to modify and metabolise fatty acids (Miles, 1992a).

Mollicutes incorporate into their membranes substantial amounts of cholesterol, notably the mycoplasmas and spiroplasmas, which need exogenously supplied cholesterol for their growth (Razin, 1992a). Membrane surface lipoproteins play a fundamental role in the biology and pathogenesis of mycoplasmas. Coat proteins, adhesins and transporters are typical mycoplasma membrane proteins, while phase variation and/or dysregulation of cytokine expression are features by which they may evade the host immune response (Razin *et al.*, 1998). Acholeplasmas are not cholesterol-dependent for their growth, but they may also incorporate this lipid into their membranes when it is present in the culture medium (Razin, 1992a). That lipoproteins are extremely abundant in the cell membrane of mycoplasmas was first shown by Dahl *et al.* (1983). This observation became apparent when the genome of a few *Mycoplasma* species was sequenced. In *M. pneumoniae,* for example, out of an estimated 150 membrane proteins, 46 open reading frames encoding putative lipoprotein genes have been identified (Himmelreich *et al.*, 1997). The lipoproteins play a role in the pathogenicity of mycoplasmas mainly by functioning as

modulins and being the major surface molecules that undergo antigenic variation. A macrophage-activating lipopeptide with a molecular mass of 2-kDa has been identified in the cell membrane of *M. fermentans* (Muhlradt *et al.*, 1996) and two lipoproteins derived from the variable lipoproteins VlpA and VlpC respectively, were identified in *M. hyorhinis* (Muhlradt *et al.*, 1998). Lipoproteins also play a major role in varying the antigenic repertoire of the mycoplasmal cell surface. The reduced genome of the mollicutes yields a minimalistic inventory of cellular components and a consequent simplicity. In addition to lacking a cell wall and being enveloped by only a cholesterol-containing unit membrane, the mollicutes are unique in having a well-defined internal cytoskleton to which shape determination and motility are attributed. The sequenced cytoskeletal genes and deduced proteins are completely unrelated to those of eukaryotes and have no known homologues in other prokaryotes (Williamson *et al.*, 1991). A 26-kDa polypeptide is a spiralin and is major constituent approximately 20 % of spiroplasmal membranes (Bove *et al.*, 1993). The mycoplasmal cytoskeltons are believed to have a role in cell-shape determination, gliding, cell division, and control of the distribution of adhesion proteins. Kahane and Schiefer, (1983) have reported that considerable quantities of magnesium and smaller amounts of calcium are also associated with mycoplasma membranes and might play a role in the stabilization of the lipid bilayer and binding to it of peripheral membrane proteins.

1.8 Pathogenicity

Mollicutes are the causative agents of a wide range of severe, respiratory, arthritic and urogenital diseases in mammals and birds. Mycoplasmas are well-established aetiological agents of acute and chronic arthritis in various animal species including porcine (*M. hyorhinis* and *M. hyosynoviae*) bovine (*M. bovis*), avian (*M. synoviae*) and murine (*M. arthritidis*), (Cole *et al.*, 1985).

Enzootic pneumonia in calves associated with *M. bovis* has been prevalent in many countries (Thomas *et al.*, 1975). The habitats of this organism in animal hosts are the mucous membrane, urogenital tracts, and gastrointestinal tracts. Those species that penetrate mucosal barriers tend to demonstrate a high degree of specificity for tissues and organs that become infected e.g. *M. mycoides*: lung and pleura, *M. arthridis*: synovial

membranes and *M. gallisepticum*: arterial walls. The aetiological agent of CBPP has been isolated quite frequently from diseased goats and sheep (Al-Aubaidi, 1972).

Ben-Menachem *et al.* (2001) have shown choline accumulation by *M. fermentans* resulted in a marked choline depletion of the growth medium and its depletion of an astrocyte cell culture induced by *M. fermentans* was associated with apoptotic death of the cells. Choline-cntaining lipids in the cell membrane of *M. fermentans* were shown to stimulate mycoplasma fusion with the eukaryotic cells, and to induce cytokine secretions by cells of the immune system. Choline-containing lipids are important mediators of tissue pathology (Rottem, 2002). Tony *et al.* (2002) have recently reported that *M. bovis* induces apoptotic death of bovine lymphocytes, indicating that lymphotoxicity is a pathogenic determinant associated with this species. *M. bovis* has also been shown to induce tumour necrosis factor α (TNF-α) and nitric oxide, two powerful initiators of immune activity (Jungi *et al.*, 1996). The interaction of mycoplasmas with macrophages and monocytes induce the production of proinflammatory cytokines, such as TNF-α, interleukins and interferon γ. Mycoplasma membrane lipoproteins and certain lipids, induce cytokine secretion by a mechanism distinct from that of bacterial lipopolysaccharides (Razin and Tully, 1995). Proteases associated with the mycoplasma membrane may damage phagocyte membrane integrity (Watanabe, 1975). The action of mycoplasma lipases or phospholipases (De Silva and Quinn, 1986) on the cell surface may reduce phagocytic function either by generating lipid hydrolysis products or by altering membrane fluidity (Ross, 1981; Thomas *et al.*, 1991). Gabridge *et al.* (1985) reported that loss of nucleic acid precursors might be particularly significant leading to inhibition of DNA and RNA synthesis in host cells tissues.

M. fermantans, M. hominis and *M. penetrans* have been isolated from individuals suffering from a variety of diseases including atypical pneumonia, urogenital infections, rheumatoid arthritis (RA) and AIDS related infections (Hays *et al.*, 1996). There is evidence that mycoplasmas may be involved as cofactors in the progress of HIV infection to AIDS (Montagnier, 1991). *M. genitalium* and *M. pneumoniae* have been isolated from the joints of patients with polyarthritis (Weinstein and Hall, 1974).

Antigenic variation of surface proteins is thought to be a survival strategy for many bacterial pathogens, including mycoplasmas (Gorton and Geary, 1997). Such pathogens

may evade host defences, thus prolonging the course of the disease or ensuring repeated infections (Mackowiak, 1984). In mycoplasmas, multigene families encode most of the phase-variable surface antigens. The presence of gene families in several *Mycoplasma* species, including *M. bovis* (Behrens *et al.*, 1994), *M. gallisepticum* (Markham *et al.*, 1993), *M. hyorhinis* (Wise, 1993), *M. pulmonis* (Bhugra *et al.*, 1995) and *M. synoviae* (Noormohammadi *et al.*, 1998) has suggested that they may be a common feature in mycoplasma genomes. Darzi *et al.* (1998) reported that *M. capricolum* subsp. *capripneumoniae* infection causes acute inflammatory response following attachment of the organism to the acinar epithelial cells. This intimate association might provide better access to nutrients adsorbed on to the host cell membrane, and the organisms may also utilise the membrane fatty acids and cholestrol. This constitutes an essential step in establishing mycoplasmal infection (Razin, 1985). The intimate association between the organism and its host cells lead to build-up in the local concentration of toxic metabolites, such as H_2O_2, which damage the host cell membrane without being rapidly catabolised by the peroxidases and catalases in the extracellular body fluids. Moreover, hydrolytic enzymes, such as proteases and phospholipases, produced by the mycoplasmas themselves, also damage the host cell membrane. Mycoplasmas take up cholestrol from the host cell membrane, thereby depleting it of an essential component (Razin, 1978). A higher concentration of malonyldialdehyde, a lipid peroxidation product, in cells infected with *M. pneumoniae* indicated that oxidation of host membrane lipids was a primary factor in triggering tissue damage (Razin, 1985). *M. hyopneumoniae* induces pneumonia by initially damaging the ciliated epithelia of the trachea, bronchi and bronchioles (DeBy *et al.*, 1993), which may be triggered by an increase in calcium uptake (Park *et al.*, 2002).

1.9 Adhesion

Mycoplasma adherence to host cells is a critical virulence mechanism. Mycoplasmas adhere to mammalian cells, compete for nutrients in the microenvironment and alter host cell metabolism (Baseman *et al.*, 1996). Without adhesion mechanisms, they would be removed by the shear forces resulting from, for example, the passage of urine, faeces or tears or the movement of mucous in the lung as a consequence of ciliary action. Tip mediated cytadherence in *M. genitalium* requires the structural and functional stability of the P140 adhesion, which is a major cytadhesin (Dhandayuthapani *et al.*, 2002). Many

pathogenic mycoplasmas of humans and animals are parasites of the respiratory and urinary tract (Kahane *et al.*, 1981). The organisms in these infections adhere to and colonise the epithelial lining of the infected organs and only rarely invade the tissues and bloodstream. These mycoplasmas can be considered to be membrane parasites. The adherence of the mycoplasmas to the epithelial cell surface is apparently the first step-in the disease process.

1.9.1 Variable surface proteins

Variable surface antigens are widely described for mycoplasmas (Razin *et al.*, 1998). It is generally believed that variable surface proteins (vsps) are a means to enhance colonisation and to adapt to the host tissue environment at various stages of infection. They have been shown to play a role in adhesion, immunomodulation, and substrate binding (Le Grand *et al.*, 1996). Vsps can undergo phase variation i.e., reversible ON/OFF switch of expression, or antigenic variation, meaning that alternative phenotypes of the protein are expressed in successive generations of the mycoplasma, thus causing a heterogeneous population. A number of different strategies to regulate phase variation have been reported for mycoplasmas. However mutant mycoplasmas deficient in adhesion molecules cannot cytadhere and are avirulent, which reinforces the critical importance of cytadherence in the mycoplasma infectious process (Baseman, 1993). The *vsp* genes of *M. bovis* seem to be regulated by site-specific DNA inversions (Lysnyansky *et al.*, 2001), and the *avg/vpma* genes for *M. agalactiae* involve gene rearrangements, although the exact mechanism is still unknown (Flitman *et al.*, 2000). A phase-variable protein *vmm* of *M. mycoides* SC which undergoes high frequency phase variation. has recently been characterised (Persson, *et al.*, 2002). The surface components involved in attachment to the host tissues, in transport of metabolites or toxic substances across the membrane, and in immune evasion are key factors for pathogenicity. The adherence of *M. hyopneumoniae* to ciliated epithelium is necessary to induce colonisation of the organism, which results in the loss of cilia (Zhang *et al.*, 1995). Thus, the adherence of mycoplasmas to host cells is an important initial step in the pathogenesis of mycoplasmal diseases.

The lack of a cell wall facilitates direct contact of the mycoplasma membrane with the specific receptors on the host cell membrane, creating a condition which could lead to

fusion of the two-membranes and exchange of membrane components. The loss of adhesion capacity by mutation results in loss of infectivity, and reversion to the cytadhering phenotype is accompanied by regaining infectivity and virulence (Krause *et al.*, 1982). In addition, there is now considerable evidence that certain mycoplasmas may have an intracellular location, for example, *M. genitalium* in monkey cells (Jensen *et al.*, 1994), and *M. fermentans* in Hela cells (Taylor-Robinson *et al.*, 1993). *M. penetrans* is known to rapidly penetrate mammalian cells and this ability is a characteristic feature of this species (Lo *et al.*, 1992).

In *M. hominis*, adhesions are found dispersed over the cell surface and are directly involved in the attachment to the host cell (Henrich *et al.*, 1993). A 55-kDa phosphoprotein detected in motile *Mycoplasma* and *Spiroplasma* species, including *M. pneumoniae* and *M. gallisepticum* may be an important constituent of the cytoskeleton (Platt and Rottem, 1990). *M. genitalium* major adhesin is an MgPa, which is an analogue of P1 (Hu *et al.*, 1987). Benedicte *et al.* (2002) have characterised a cytadhesion P40 for *M. agalactiae*. It is involved in the adhesion of *M. agalactiae* to lamb synovial membranes cells. P40 protein was shown to be located in the membrane by Triton X-114 partitioning experiments which is an inherent property related to its function as an adhesin (Razin *et al.*, 1998).

1.10 Oxidative damage

M. pneumoniae, growing on solid media, showed clear beta haemolysis of mammalian erythrocytes (Somerson *et al.*, 1963). The haemolysin produced by *M. pneumoniae* is hydrogen peroxide (H_2O_2), which would damage tissue of the respiratory tract, and hence play a role in pathogenicity (Somerson *et al.*, 1965). H_2O_2 production by *M. mycoides* subsp. *mycoides* during glucose and glycerol oxidation *in vitro* has led to speculation concerning the role of oxidative damage in pathogenesis (Miles *et al.*, 1991). The cytopathogenic effects of *M. mycoides* subsp. *capri* in tracheal organ culture was probably caused by H_2O_2 (Cherry and Taylor–Robinson, 1970). So it is assumed that mycoplasma virulence may be increased by the production of reactive oxygen species. Many biochemical processes including general membrane toxicity (Gabridge and Murphy, 1971), phospholipases and ATPases have been suggested as the cause of epithelial membrane cytotoxicity induced by

pathogenic mycoplasmas. H_2O_2 and superoxide radicals have been incriminated as causing oxidative damage to the host cell membrane (Almagor *et al.*, 1984; Figure 1.2).

Moreover, adhesion may enable oxidative damage of host cells by facilitating the transfer of metabolites such as peroxides and superoxides from the mycoplasmas to the host cells (Razin and Jacobs, 1992; Vilei and Frey, 2001). Kanan and Baseman (2000) reported haemolytic and haemoxidative activity in *M. penetrans* and haemoxidation is directly linked to mycoplasma-generated H_2O_2. Oxidative killing involves an NADPH-oxidase, which is assembled in the phagosomal membrane and converts oxygen to superoxide (O_2^-) when neutrophils ingest the microorganisms (Clark, 1999). The increased production of O_2^- and H_2O_2 can lead, under certain conditions, to DNA damage (Bolan and Ulvik, 1990).

H_2O_2 is produced during the oxidation of glucose, glycerol and other substrates by fermentative *Mycoplasma* species. However where substrates are oxidised via pyruvate to acetate and carbon dioxide (*M. bovis* and *M. agalactiae*), NAD^+ is reduced and the NADH formed is then oxidised by NADH oxidase to regenerate NAD^+. NADH oxidase reduces molecular oxygen directly. Due to this reduction of oxygen there is a series of single electron transfers, which may generate reactive intermediates such as O_2^- and OH^-. In this way H_2O_2 is formed. In many *Mycoplasma* species the major product may be H_2O. In addition to NADH, L-α-glycerophosphate (GP) oxidase has been identified as a H_2O_2-producing enzyme (Miles *et al.*, 1991). The significance of H_2O_2 and O^-_2 production is indicated by the presence of catalase and superoxide dismutase (SOD), (Fridovich, 1976) and SOD is a manganese-dependent enzyme, which dismutates O_2^- to H_2O_2 and O_2.

$$2\,O_2^- + 2H^+ \xrightarrow{\text{Superoxide dismutase (SOD)}} H_2O_2 + O_2$$

Reports of catalase and SOD activity in mycoplasmas are conflicting (Lynch and Cole, 1980) and it seems unlikely that they have sufficiently high levels of such enzymes to avoid damage by phagocyte oxygen radicals. H_2O_2 formed by SOD or by flavin linked oxidases (Brunori and Rotilio, 1984) may be decomposed by catalase, an enzyme that catalyses the H_2O_2 into water and molecular oxygen.

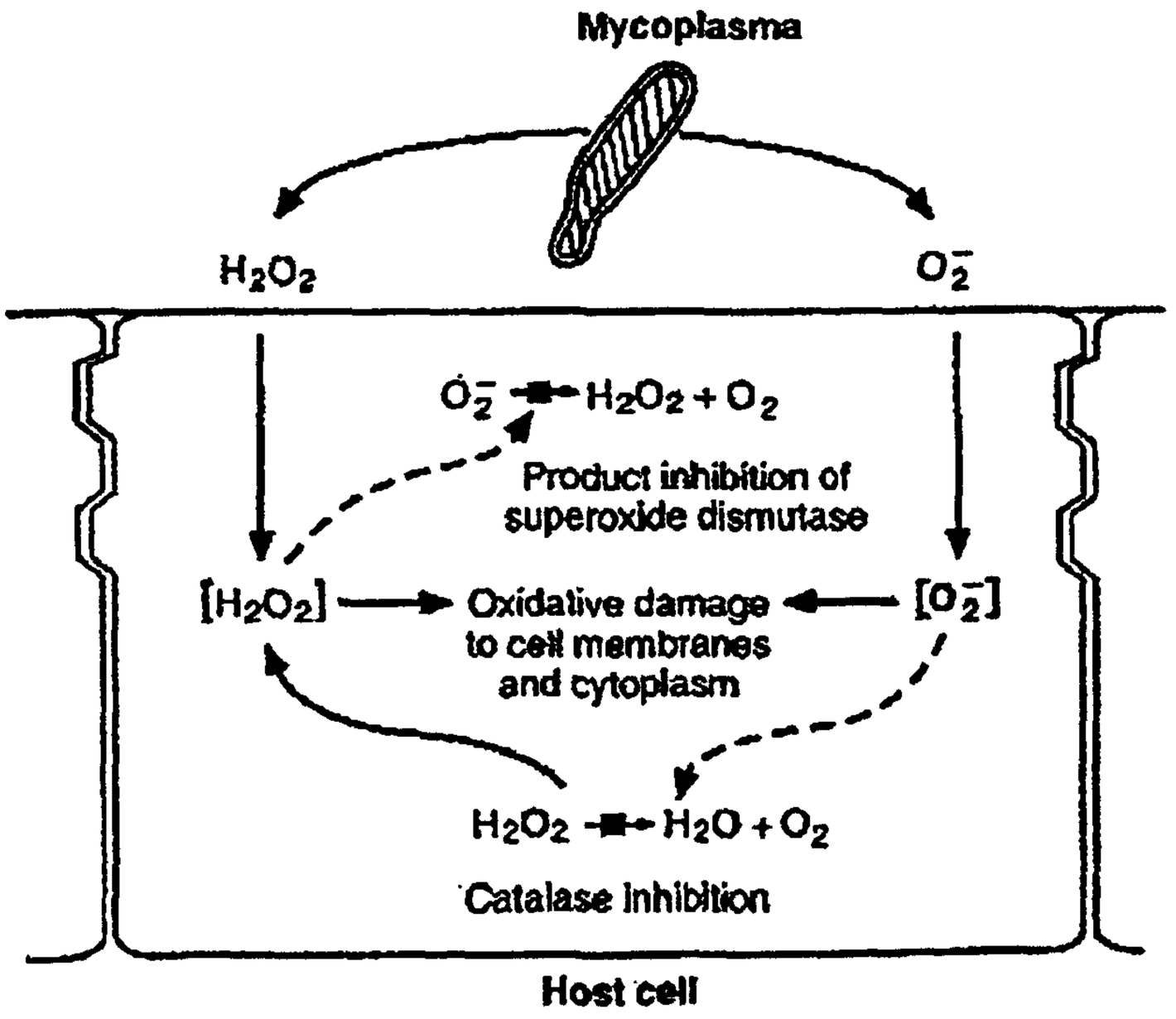
$$2H_2O_2 \xrightarrow{\text{Catalase}} 2H_2O + O_2$$

Catalase is necessary in suspension media since many *Mollicutes* produce substantial quantities of H_2O_2 leading to reduced metabolic activity and/or cell viability. The presence of catalase also simplifies data analysis using the O_2 uptake method, since in the absence of catalase; oxygen might be reduced to either H_2O or H_2O_2 (Miles *et al.*, 1991).

Mycoplasmas produce H_2O_2 but they lack catalase to breakdown these toxic products. This is supported by genome sequence studies which have shown that *M. pneumoniae* and *M. genitalium* specifically lack genes dealing with oxidative stress, including those encoding catalase, peroxidase and SOD (Fraser *et al.*, 1995; Himmelreich *et al.*, 1996). *Mycoplasma* species appear not to produce catalase (Weibull and Hammarberg, 1962) or SOD (Lynch and Cole, 1980). Production of ammonia by mycoplasmas has also been proposed as a virulence factor (Stalheim and Gallagher, 1977).

Arginine deiminase may also have a role in pathogenicity. Arginine utilising mycoplasmas are toxic to mammalian cells in culture due to arginine depletion from the culture medium, an effect that may be reproduced by mycoplasma cell extracts or arginine deiminase. In *M. arthritidis,* multiple metabolic roles for arginine deiminase were suggested by the presence of two cytoplasmic forms (Weickmann and Fahrney, 1977). Matsuura *et al.* (1990) have also proposed that NH_3 produced by the arginine dihydrolase pathway may be primarily responsible for the tissue damage following the intracutaneous inoculation. However, Ben-Menachem *et al.* (1997) suggested that a thioredoxin reductase system, identified in certain *Mycoplasma* species, might provide some protection from reactive oxygen compounds. In culture media, reactive oxygen compounds will be inactivated by serum catalase and SOD. These enzymes are also widely distributed in host tissues. *In vivo*, however, the production and release of H_2O_2, O_2^- and other reactive species (OH^-), may be particularly significant because of the close adherence of mollicute cells to host cell membranes. Stewart *et al.* (1994) reported that the nucleases of mollicute combined with O_2^- induce clastogenic effects in cell cultures, and were reduced by the presence of antioxidants, SOD and free radical scavengers such as mannitol.

Figure 1.2. Mechanism of oxidative damage to host cells by adhering *M. pneumoniae* by increasing concentration of H_2O_2 and O_2^- (Almagor *et al.*, 1984)



1.11 Toxin production

Finlay *et al.* (1938) reported that *M. neurolyticum* produces a toxin, which causes "rolling disease" in mice and when injected intracerebrally into mice, induced a characteristic disease. *M. gallisepticum* also produces a toxin similar to the disease caused by *M. neurolyticum* (Cordy and Adler. 1957), young turkeys poults injected intravenously with an early passage broth culture of *M. gallisepticum.* The turkeys develop symptoms of encephalitis associated with vasculitis and foetal necrosis in brain arteries. The neurotoxicity of concentrated organisms required intact viable mycoplasmas: heating the mycoplasma suspension at 50°C for 1 hour or repeated freezing and thawing destroyed the toxic activity.

Galactan has been associated with the pathogenicity of *M. mycoides* subsp. *mycoides.* Galactan is a toxin or is a component of a soluble toxin, and is capable of causing necrosis and a striking connective tissue response in cattle in the absence of mycoplasmas (Buttery *et al.,* 1980). This toxin was one of the first toxic products associated with pathogenic mycoplasmas (Plackett and Buttery, 1958). This galactan was prepared from culture supernatant of *M. mycoides* (Buttery *et al.*, 1976). *Mycoplasma fermentans* possesses a toxic factor that is associated with both viable and lysed cells (Gabridge and Murphy, 1971).

An inflammatory toxin of *M. bovis* increases vascular permeability and activates complements. A toxic product from *M. bovis* was extracted with aqueous ethanol (Geary *et al.,* 1981). The complex polysaccharide was composed of glucose, glucosamine or galactosamine and a heptose but did not contain significant amount of protein or fatty acids. *M. fermentans* is capable of inducing lethal toxicity syndromes similar to endotoxic shock, when injected into mice in high doses (Gabridge *et al.*, 1972). It appears that pathogenic mollicutes rarely produce toxins having specific and direct effects on host cells. However, mollicutes produce low molecular weight compounds, such as H_2O_2 and ammonia, which may have toxic effects upon the host cells.

1.12 Capsule production

Whitfield (1988) has reported that capsules, generally composed of high molecular weight polysaccharides, which are widely distributed in bacteria. They are generally considered to promote pathogenicity by enabling bacteria to bind irreversibly to negatively charged surfaces (Robb, 1984), by toxic effects, or by promoting resistance to phagocytosis (Rosenbusch and Minon, 1992). Protection is provided where cell aggregates are surrounded by a common glycocalyx of exopolysaccharide material. This increases resistance to degradative enzymes (Isenberg, 1988), but it is not clear whether such aggregates are commonly found in mollicutes. Capsules have not been described among genera of *Acholeplasma, Spiroplasma, Aneroplasma*, or *Asteroplasma.* Lipoglycan have commonly been found in members of these mollicutes but these structures are considered integral to the mycoplasmal membrane. Electron microscope images, of lipoglycan when stained with ruthenium red, are of lower molecular weight than capsule, and require hot phenol extraction for their separation from other membrane components (Smith, 1984). Marshall (1992) showed that mutant strains of *M. mycoides* subsp. *mycoides*, which produced reduced quantities of capsular polysaccharide, had an increased susceptibility to phagocytosis in an *in vitro* assay. Jones *et al.* (1972) have also shown that the removal of *M. pulmonis* capsule-associated protein, using trypsin, also increased susceptibility to phagocytosis *in vitro*. Some mollicutes including *M. mycoides* subsp. *mycoides*, possess a capsule-like structure. Ruthenium red staining of the. *M. mycoides* subsp. *mycoides* capsule revealed an amorphous layer of up to 30 nm in thickness (Howard and Gourlay, 1974). In infected porcine lung tissue, the *M. hyopneumoniae* capsule can extend up to 40 nm (Tajima and Yagishahi, 1982). Chemical analysis indicated that the capsule was composed of a galactan, a polymer of the monosaccharide galactose (Buttery and Plackett, 1960). The polysaccharide capsule and oxidative damage from H_2O_2 production may play important roles in CBPP infection (Lloyd *et al.*, 1971; Miles *et al.*, 1991). Capsules are generally considered to contribute to pathogenicity by promoting binding to host tissue surfaces and enhancing resistance to phagocytosis. In addition there is evidence that the capsule of *M. mycoides* SC might have a direct toxic effect on host cells and its structural similarity to bovine pneumogalactan further suggests that it might induce auto-immune reactions. Henderson and Miles (1990), using chemostat continuous culture to control growth rate showed that capsule synthesis in *M. mycoides* SC strain T_1 increased with

increasing glucose concentration. Plackett *et al.* (1963) reported that the major carbohydrate in the Australian strains V5 and Gladysdale was galactose (> 90 %).

In contrast the capsule of *M. dispar* has been shown to be composed of a polymer of galacturonic acid (Rosenbusch *et al.*, unpublished data). Polysaccharides isolated from *M. mycoides* subsp. *capri* (Jones *et al.*, 1965), were recognised as glucan, a polymer of glucose. Geary *et al.* (1981) reported that *M. bovis* is known to produce a polysaccharide, composed of glucose, glucosamine or galactosamine and heptose that act as an inflammatory toxin. *M. capripneumoniae* also produces similar extracellular polysaccharides, consisting of four neutral sugars (glucose, galactose, mannose and fructose) and two amino sugars (glucosamine and galactosamine) but their role in disease is not known (Rurangirwa *et al.*, 1987).

1.13 Enzyme activities

Extracellular degradative enzyme activities are frequently associated with pathogenicity in cell-walled bacteria. The enzymes most commonly involved are proteases, amino peptidases, collagenases, lipases, nucleases, glycosidases and phospholipases. Simecka *et al.* (1992) reported that an experimental infection in cattle with *M. bovis* resulted in the destruction of cartilage and development of fibrotic lesions within joints. *M. orale* has been shown to induce collagenase activity in infected cell cultures and such induction may be important in the development and persistence of connective tissue disease (Kulve *et al.*, 1981). A whole array of potent hydrolytic enzymes has been identified in mycoplasmas (Shibata *et al.*, 1995). Most remarkable are the mycoplasma nucleases that may degrade host cell DNA (Paddenberg *et al.*,1998).

Proteolytic activity is found in different *Mycoplasma* species (Alutto *et al.*, 1970). Liquefaction of coagulated serum by *M. capricolum* and by *M. mycoides* subsp. *mycoides* was also observed (Tully *et al.*, 1974). Czekalowski *et al.* (1972) screened many strains for gelatinolytic activity and only *M. arthritidis* liquefied gelatine. A number of reports have shown that mycoplasma contain peptidase activity. Aminopeptidase has been demonstrated in *U. urealyticum, M. pneumoniae, M. hominis* and *M. fermentans, M. bovirhinis, M. dispar* and *M. bovis* (Neil and Ball, 1980). Chooules and Gray (1971)

showed that membranes of *A. laidlawii* contained exopeptidase activity. Davis *et al.* (1987) have reported pyrophosphatase activity in *U. urealyticum.* Glutamate dehydrogenase (GDH) activity was reported in some mycoplasmas (Smith, 1971) but not in *U. urealyticum* (Smith *et al.*, 1992). Two enzymes, α-glucosidase and ornithine transcarbamylase, were present in *M. mycoides* LC, but not in SC strains (Salih *et al.*, 1983). However phosphopentomutase activity has not been reported in *M. pneumoniae* and *M. genitalium* but was reported in *U. urealyticum* (Cocks *et al.*, 1985). The enzyme was not seen in three human *Mycoplasma* sp. but was in *M. pulmonis* (Chambaud *et al.*, 2001). Johnson and Pitcher (2000) have reported the presence of ecto 5'-nucleotidase activity on *Mycoplasma* species. Glucosidases are potential virulence factors, since they may affect host cell structures, so triggering autoantibody production. They are needed in mollicutes for the processing of cellular polysaccharides and glycocojugates including glycolipids, glycoproteins and lipoglycans (Smith, 1979), however significant glucosidase activity in a range of *Mycoplasma* species has not been detected (Miles, 1992b).

Pollack (1975, 1979) found that the NADH oxidase activity of *Acholeplasma* was localised in the membrane fraction except in *Acholeplasma multilocale* PN525T, which had low levels of NADH oxidase activity in its cytoplasm fraction, and no activity was detected in the membrane fraction. NADH oxidase activity is localised in the membrane of almost every prokaryote. *Acholeplasma* species but not *Mycoplasma, Spiroplasma* and *Ureaplasmas* species also have these characteristics (Pollack, 1986). However unlike mycoplasmas, *U. urealyticum* lacks both NADH oxidase activity (Pollack *et al.*, 1997) and an NADH oxidase gene.

Lipolytic activity is found in many mollicutes and shown by their ability to form films and spots on serum agar medium and to cause clearing zones on egg yolk agar (Freundt, 1983). Lipase activity is important in pathogenicity. Microbial strains of the same genus or species may produce different lipases (Taipa *et al.*, 1992) that can be exploited in the pharmaceutical and food industries. Bhandari and Asnani (1989) reported a phospholipase A_2 that causes lysis of mammalian erythrocytes; it has also been demonstrated in culture supernatants of *M. mycoides* and *A. laidlawii* and lysophospholipase activity in *M. gallisepticum* (Gatt *et al.*, 1982). Smith (1979) reported that glycosidases are required in

mollicutes for the possession of cellular polysaccharides and glycoconjugates including glycolipids, glycoproteins and lipoglycans.

1.14 Growth requirements and growth media

Mollicutes are nutritionally exacting and need to be supplied with a large array of precursors for the synthesis of macromolecules. The known growth requirements of *A. laidlawii* strain B and *M. mycoides* strain Y are listed in Table 1.3. Both organisms require a vast range of amino acids, nucleic acid precursors, lipid precursor molecules and vitamins. *M. mycoides* strain Y may obtain certain amino acids from peptides and by degradation of bovine serum albumin (BSA) (Rodwell, 1983). Media for their growth contain peptone, beef heart infusion, DNA to supply nucleic acid precursors, yeast extract and animal serum as a non-toxic source of lipids. Miles (1992a) reported that the requirements of mollicutes for inorganic salts (K^+, Mg^{2+} and PO_4^{-3}) are presumably comparable to those of cell-walled bacteria, but have not been studied in detail. Yeast extract provides a variety of nutrients including nucleotides, vitamins and mineral salts. Fresh yeast extract is superior to commercial dehydrated extracts. Other requirements include: NADH, a coenzyme also present in animal tissue and yeast extract; and L-cysteine hydrochloride, which acts as a reducing agent, lowering the oxidation-reduction potential of the medium and making it more suitable for the growth of anaerobic or microaerophilic organisms (Miles, 1992a). The role of NaCl is to increase medium tonicity, needed because mollicutes lack a rigid cell wall and are therefore more susceptible than cell-walled bacteria to lysis in hypo-osmotic media

Mycoplasmas grow best with an osmotic pressure range from 10-14 atmosphere (Leach, 1962) and are more resistant to physical stresses in broth than in distilled water or phosphate-buffered saline (Smith and Sasaki, 1958). A deficiency of glycerol, cholesterol or fatty acids results in rapid death and lysis of the *M. mycoides*. This was attributed to unbalanced growth in which the synthesis of lipids ceased but cytoplasmic synthesis continued (Rodwell and Abbot, 1961). Glycerol, cholesterol and long chain fatty acids are all needed for the synthesis of an undetermined cell component, which is necessary for the structural integrity of the cell. Sterol, which makes up some 20 % of the membrane lipids, is required by all mollicutes except for species of *Acholeplasma* and *Asteroplasma*.

Cholesterol is the sterol usually provided in the medium, but others such as cholesanol and ergosterol can be substituted (Rodwell and Mitchell, 1979). Glycerol is required by *M. mycoides* for the synthesis of L-α-glycerophosphate and thus glycerides. In addition to long chain fatty acids, most mycoplasmas require a sterol for growth, a nutritional dependance not found elsewhere among prokaryotes (Edward and Fitzgerald, 1951). Cholesterol functions as a regulator of membrane fluidity, maintaining an intermediate fluid condition during changes in growth temperature, or following alterations in the fatty acid composition of membrane lipids (Rothman and Engelman, 1972). The effect of cholesterol on ion transport, control of osmotic fragility (Rottem and Verkleij, 1982), and the activity of membrane-bound enzymes (McElhaney, 1982) has been attributed principally to cholesterol-mediated alterations in the physical and lipid environment of the respective proteins. It has long been suggested that cholesterol increases the tensile strength of the cell membrane of mycoplasmas, thus facilitating their survival and growth without the protection of a rigid cell wall. The presence of serum proteins to bind and reduce the toxicity of fatty acids is also important (Miles, 1992a). The nutritional properties of serum vary by animal source, possibly reflecting differences in lipid concentrations, but other differences may be important. For example bovine foetal serum contains relatively high concentrations of fructose (3mM, Miles *et al.*, 1985) and horse serum is rich in urea (Shephard and Masover, 1979). Serum is usually inactivated at 56°C for one hour before addition to the medium because it reduces mycoplasmacidal complements contained in the serum.

The growth of *M. mycoides* species in medium containing serum is markedly affected by the nature of the sugar source. Glucose supported higher growth rates than other sugars, even though other sugars were present in saturating concentrations (Miles *et al.*, 1986). It is therefore apparent that glucose may have a role in metabolism, perhaps in the regulation or synthesis of other sugars, in addition to providing an energy source. *M. arthritidis* can derive sufficient energy for the synthesis of macromolecules from the metabolism of arginine via arginine dihydrolase (Schimke *et al.*, 1966). Thus the maintenance of an adequate supply of arginine is important for continued growth of those organisms possessing the arginine dihydrolase pathway.

Mollicutes generally grow and survive over a fairly narrow pH range. For most species, the optimum pH is approximately 7.4 and growth occurs between about pH 6.5 and 8.0 and decrease in pH to less than 6.5 causes cessation of growth followed by rapid death of cells (Rodwell and Mitchell, 1979). Ureaplasmas are exceptional, having an optimum pH of 6.0 to 6.5 and being inhibited above pH 7.5 (Razin *et al.*, 1977). Mollicutes have a narrow range of pH values; therefore media should be well buffered. The buffers most commonly used are phosphate buffer and HEPES (Miles, 1992a). Oxygen causes an increase in the growth rate of certain mycoplasmas (Rodwell and Mitchell, 1979) by increasing the rate of pyruvate oxidation and thus the yield of ATP during the metabolism of glucose or other carbohydrates (Miles *et al.*, 1988; Taylor *et al.*, 1994). In general most mollicutes grow better in aerobic or microaerophilic (10 % v/v oxygen) conditions with enhanced CO_2 levels (10 % v/v). Mycoplasmas have insufficient energy yielding metabolism so must consume large amounts of substrate to maintain an adequate supply of energy for macromolecule synthesis. The addition of 0.5 % (w/v) glucose to undefined media is sufficient to allow maximum growth of fermentative mycoplasma. Arginine 0.2 % (w/v) can be a major source of energy for some species of *Mycoplasma*.

All species of *Mycoplasma, Ureaplasma* and *Spiroplasma* require sterol for growth and are incapable of synthesising long chain fatty acids. Serum is usually added at a concentration of 10-20 % (v/v): horse, bovine, and swine sera are commonly used (Rodwell, 1983). Serum has been shown to provide, among other nutrients, fatty acids and cholestrol in an assimilable nontoxic form. Tween-80 at a final concentration of 0.04 % (v/v) is essential for the growth of the sterol non-requiring mycoplasmas (Rose *et al.*, 1993). Most mycoplasmas cannot synthesise any fatty acid and therefore depend on the host for their supply. They synthesise their own membrane phospholipids and glycolipids from exogenously supplied fatty acids. *Mycoplasma* sp. can be cultured in cell-free media, have limited biosynthetic abilities require complex media for growth, including amino acids, nucleic acid precursors, lipids, vitamins, inorganic ions and glucose as an energy source. Certain strains grow poorly or not at all in artificial media (Razin and Freundt, 1984). Desantis *et al.* (1989) have emphasised that the importance of 2-deoxyribose 5-phosphate adolase activity in mollicutes, which may enable the utilisation of DNA as a carbon and energy source for growth. *M. genitalium* and *M. pneumoniae* lack all the genes involved in amino acid synthesis, making them totally dependent on the exogenous supply of the

complete spectrum of amino acids (Fraser *et al.*, 1995; Himmelreich *et al.*, 1996). A problem in formulating defined media is the occurrence of amino acid antagonisms; i.e. two or more amino acids competing for the same transport system. Such amino acids must either be made available in favourable proportions or one must be provided in the form of a suitable peptide that is taken up by an independent system. For example, *M. mycoides* may take up alanine in the form of a di-or tri-peptide (Rodwell, 1983).

There have been attempts to develop defined media for mollicutes, based on tissue-culture media. The most successful applications of this type of medium are those developed for spiroplasmas. H1 medium was developed for the growth of *S. pirum* (Hackett *et al.*, 1987), but was not completely defined. Hackett and Lynn (1985) reported that defined media for spiroplasmas, mainly based on cell tissue culture media have more than eighty organic compounds. The role of the majority of these compounds in the nutrition of spiroplasma however, is unknown. Anaeroplasmas may also be grown in defined media containing glucose, amino acids, vitamins, volatile fatty acids, phophatidyl choline, cholesterol and minerals (Robinson, 1979). Thus the mollicutes growth media used are universally complex.

1.15 Energy sources

Glucose and other sugars are the main energy sources for the mollicutes. Some species of *Mycoplasma* and *Spiroplasma* may obtain energy from the hydrolysis of arginine. Others which do not oxidise sugars or hydrolyse arginine may obtain energy from oxidation of organic acids (Miles *et al.*, 1988). Anabolism involves the formation of cellular structures and macromolecules. The precursors in the form of nucleotides, amino acids, fatty acids and coenzymes are all derived from central or core metabolites (Neidhardt *et al.*, 1990).

M. genitalium and *M. pneumoniae* depend mostly on glycolysis as a means of synthesising ATP. Sequencing of the entire genome of some mycoplasmas has identified genes that encode the components of the pyruvate dehydrogenase complex, phosphate acetyl transferase and acetate kinase, as well as a deficient pentose phosphate pathway (Fraser *et al.*, 1995; Himmelreich *et al.*, 1996). Mollicutes lack a complete tricarboxylic acid cycle and have no quinones or cytochromes, ruling out oxidative phosphorylation as an ATP

generating mechanism (Miles, 1992b; Pollack *et al.*, 1997). Members of the fermentative group produce acids from carbohydrates, decreasing the pH of the growth medium. The sequences of *M. genitalium* and *M. pneumoniae* genomes showed that these mycoplasmas carried all the enzymes of Embden Meyerhof parnas (EMP) pathway. Pyruvate generated by glycolysis can be further metabolised either to lactate by lactate dehydrogenase or, to acetyl coenzyme A (acetyl-CoA) by the pyruvate dehydrogenase pathway (Fraser *et al.*, 1995; Himmelreich *et al.*, 1996). Fermentative mycoplasmas oxidised sugars and produce acetate and CO_2 aerobically and anaerobically lactate, acetate and CO_2.

Some non-fermentative mollicutes and some fermentative species possess the arginine dihydrolase pathway. Arginine hydrolysis by this pathway results in the production of ornithine, ATP, CO_2 and ammonia, raising the pH of the culture medium (Razin, 1978). Some mycoplasmas metabolise neither sugars nor arginine but are capable of oxidation of organic acids to acetate and CO_2. Pyruvate metabolism genes were identified in some mycoplasmas, being clustered in *M. capricolum* (Zhu *et al.*, 1994). Nonglycolytic *Mycoplasma* sp. apparently lack a functional hexose monophosphate shunt (HMS) and also lack the two access points from the HMS to the Embden Meyerhof Parnas (EMP) pathway; they have no phosphofructokinase (PFK) or aldolase activities. The metabolic needs of these nonglycolytic strains, therefore, require direct entry to the C carbon arm of the EMP pathway at the triose level or alternate modes of ATP synthesis (Bridger and Henderson, 1983). Non-fermentative *Mycoplasma* species, such as *M. agalactiae*, *M. bovis* and *M. bovigenitalium* oxidise organic acids (lactate and pyruvate) and in these species the pathway from pyruvate to acetate plus CO_2 would appear to be present.

Energy in mycoplasmas may be obtained by the fermentation of sugars (via pyruvate to lactate) the partial oxidation of organic acids, e.g. lactate or pyruvate, to acetate plus CO_2 and the metabolism of arginine by the arginine dihydrolase pathway to ornithine, NH_3 and CO_2 (Pollack, 1992). In *M. mycoides* glucose is transported into cells by the phosphoenolpyruvate:phosphotransferase system (PEP:PTS) (Cirillo, 1979) and is further metabolised by the EMP to pyruvate (Cocks *et al.*, 1985; Miles, 1992b; Figure 1.3).

Under anaerobic conditions, pyruvate is reduced to lactate and there is a yield of 2 mol of ATP per mol of glucose.

Table 1.3 Essential constituents of defined and semi-defined media for *M. mycoides* strain Y and *A. laidlawii* strain B (Rodwell and Mitchell, 1979; Miles, 1992a).

Inorganic ions	Amino acids	Saccharide	Lipid and precursors	Vitamins and coenzymes.	Nucleic acid precursors
***M. mycoides* strain Y**					
K^+ Mg^{+2} PO_4^{-3}	Alanine, arginine, asparagine, cysteine, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenyl-alanine, proline, serine, threonine, tyrosine, tryptophan, valine	Glucose	Fatty acids, glycerol, sterol	Coenzyme A riboflavin, nicotinic acid, thiamine, pyridoxamine, α-lipoic acid	Guanine, uracil, thymine
***A. laidlawii* strain B**					
K^+ Mg^{+2} PO_4^{-3}	Alanine, arginine, asparagine, cysteine, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, proline, serine, threonine, tryptophan valine	Glucose	Unsaturated fatty acids	Biotin, folic acid, nicotinic acid, pantetheine, pyridoxal, riboflavin and thiamine	Adenine, cystidine, guanosine

But under aerobic conditions, pyruvate is converted via phosphate acetyltransferase and acetate kinase activities to acetate and CO_2 (Kahane *et al.*, 1978) and gives an additional 2 mol of ATP per mol of glucose. This partial oxidation of pyruvate leads to the reduction of NAD^+ to NADH. NAD^+ is regenerated by NADH oxidase activity. Individual species may use one or any combination of these reactions to obtain energy, enabling the subdivision of the genus into major physiological groups (Miles *et al.*, 1994). Amongst fermentative mycoplasmas, *M. bovirhinis*, *M. fermentans*, *M. pullorum* and a number of other species have been shown, unlike *M. mycoides*, to be unable to oxidise lactate and pyruvate (Miles *et al.*, 1991). Many non-fermentative arginine-hydrolysing mycoplasmas are unable to oxidise organic acids. *M. verecundum* (non-fermentative arginine-hydrolysing mycoplasma) lacks organic acid oxidising ability and its energy sources are unknown (Miles *et al.*, 1994).

Acholeplasmas possess the enzymes of the EMP pathway and also appear to possess a fully functional pentose phosphate pathway, with the exception of *A. parvum* (De Santis *et al.*, 1989). Spiroplasmas possess all the key enzymes of the EMP pathway (Pollack *et al.*, 1989). In anaerobic mollicutes, the enzyme activities of the EMP pathway and the nonoxidative portion of the EMP pathway have been demonstrated (Petzel *et al.*, 1989).

The overall reaction of the EMP pathway in *M. mycoides* is,

$$\text{Glucose} + 2\ \text{ADP} + 2\ NAD^+ + 2\ \text{Pi} \longrightarrow 2\ \text{pyruvate} + 2\ \text{ATP} + 2\ \text{NADH}$$

This reaction is followed by:

$$2\ \text{Pyruvate} + NAD^+ \longrightarrow 2\ \text{Lactate} + 2\ NAD^+ \text{(anaerobic)}.$$

or:

$$2\ \text{Pyruvate} + 2\ NAD^+ + 2\ \text{ADP} + 2\text{Pi} \longrightarrow 2\ \text{Acetate} + 2\ CO_2 + 2\ \text{NADH} + 2\ \text{ATP}.$$

(aerobic).

The utilisation of energy substrates at high rates may reduce substrate availability to host cells and result in the formation of toxic products particularly H_2O_2 production from carbohydrate metabolism (Miles *et al.*, 1991).

Arginine is an energy source for non-fermentative mycoplasma. The fermentation of ATP proceeds by the arginine dihydrolase pathway (Weckmann and Fahrney, 1977). Arginine is catabolized in *Mycoplasma* and *Spiroplasma* species by the arginine dihydrolase pathway (Razin, 1978).

$$\text{Arginine} + H_2O \xrightarrow{\text{Arginine deiminase}} \text{Citrulline} + NH_3$$

$$\text{Citrulline} + \text{Pi} \xrightarrow{\text{Ornithine transcarbamylase}} \text{Ornithine} + \text{Carbamyl phosphate}$$

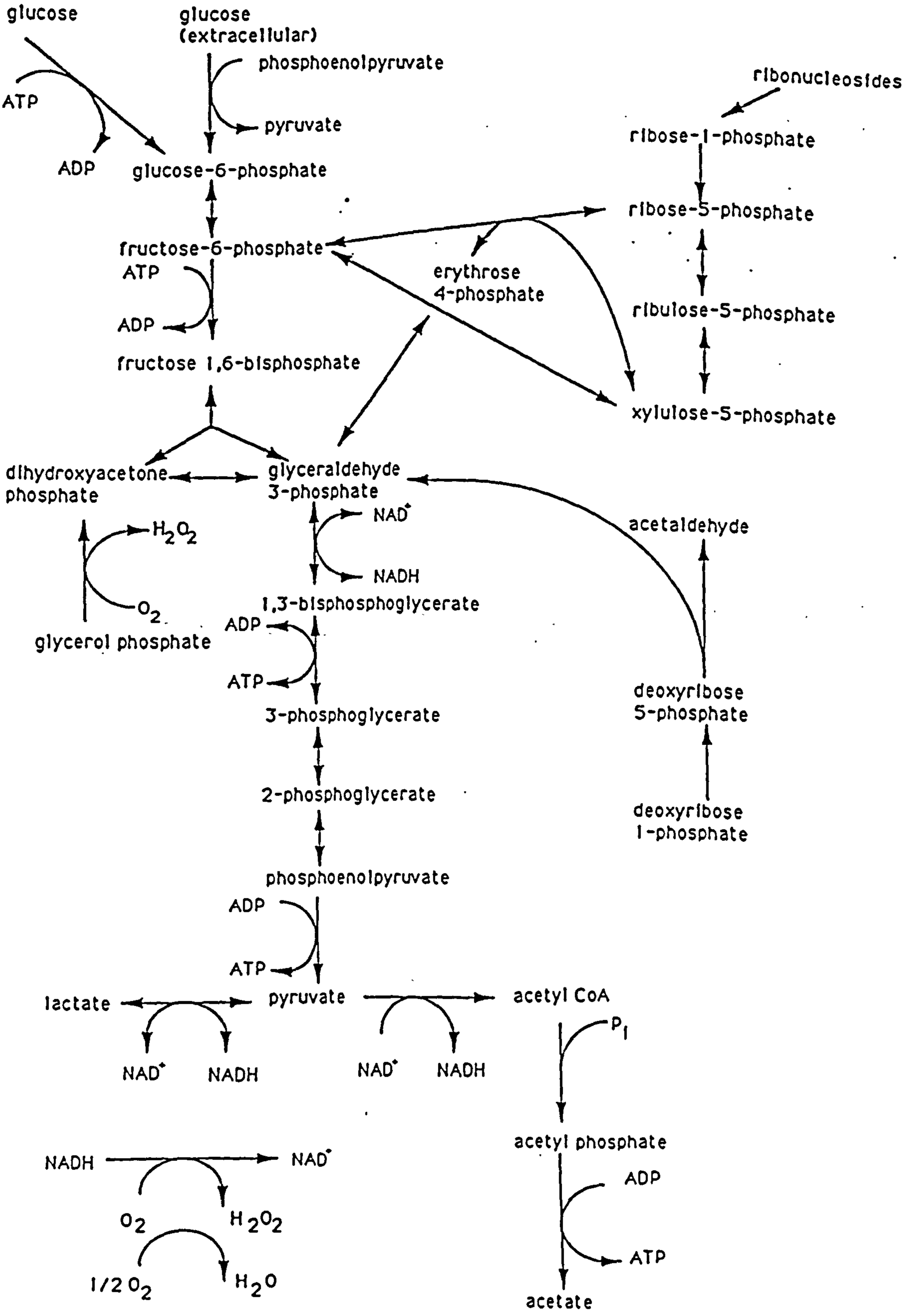
$$\text{Carbamyl phosphate} + \text{ADP} \xrightarrow{\text{Carbamate kinase}} \text{ATP} + CO_2 + NH_3$$

DeSantis *et al.* (1989) reported that arginine would appear to be the sole energy source in the majority of mycoplasmas possessing this pathway and in these organisms key enzymes such as aldolase of the EMP pathway are absent. A number of arginine-utilising strains also ferment sugars for example *M. fermentans* and *M. penetrans* (Olson *et al.*, 1993). Phosphorolysis of citrulline occurs by a catabolic ornithine transcarbamylase. The latter transfers its energy-rich phosphate bond to ADP, yielding ATP, CO_2 and NH_3 (Poolman *et al.*, 1987). *U. urealyticum,* like all ureaplasmas, is unique as they generate 95 % of their ATP through hydrolysis of urea by urease (Smith *et al.*, 1993). Urease activity appears essential for growth since this is inhibited by the specific urease inhibitor fluroamide (Blanchard *et al.*, 1988). Growth is also dependent on the exogenous supply of urea.

1.16 Transport systems

Bacteria have evolved mechanisms which allow them to adapt rapidly and respond to variation in their environment.

Figure 1.3 Catabolism of carbohydrate in *M. mycoides* (Cocks *et al.*, 1985; Miles, 1992b).



Certain substrates may be preferred to less energetically favourable ones. The key to such metabolic flexibility is the regulation of synthesis or activity of transport systems. Nutrients enter microorganisms by the activity of specific membrane proteins, by facilitated diffusion or ion gradient. The specific diffusion pore mediates translocation of hydrolytic compounds through the cytoplasmic membrane by a protein-mediated simple diffusion process (Sanno *et al.*, 1968). PEP:PTS catalyses the first step in the utilisation of some sugars by many types of bacteria. This system is found in many eubacteria but is absent from archea and eukaryotes. The sugar taken up inside the cell is phosphorylated and form sugar phosphate and pyruvate. The general PTS proteins which are required for the transport of carbohydrates are proteins such as enzyme I (EI), heat stable protein (HPr), the enzyme II (EII) complex which involves the recognition of the various sugars in the medium (Postma, 2000). Vincet and Razin (1973) have reported sugar transport in mycoplasmas by PTS. Phosphorylation is a general and fundamental regulatory process in both prokaryotic and eukaryotic cells. Protein kinases catalyse the transfer of a phosphate from ATP to a specific amino acid residue on a protein, altering a charge of the site with a consequent change in protein conformation.

Three types of transport systems were found in *M. genitalium* and *M. pneumoniae*. The first is the ABC transport system, consisting of two ATP binding domains, two membrane-spanning and one substrate-binding domain. The second type consists of the PTS system for transporting sugars, resembling homologous systems of gram-positive bacteria. The third is facilitated diffusion by transmembrane proteins (Fraser *et al.*, 1995; Himmelreich *et al.*, 1996). ABC transporter systems are involved in import or export of a large variety of substrates, including sugars, peptides, proteins and toxins. The ABC transporters were the most frequent class of proteins found in *B. subtilis* and in *E. coli* (Blattner *et al.*, 1997).

The uptake and metabolism of carbon sources constitutes a fundamental requirement for growth and development of bacteria. Numerous mechanisms have evolved to cope with this demand. One particularly widespread mechanism amongst bacteria is the PEP-dependent PTS (Postma *et al.*, 1993). The energy stored in the high-energy phosphate bond in PEP is harnessed to drive the translocation of phosphate of the substrate, with its concomitant to drive the translocation trapping the product with in the cell. The complete PTS is composed of both soluble and membrane-bound proteins (Mitchell *et al.*, 1991).

Glass *et al.* (2000) identified 28 different transporters in *U. urealyticum*. In mollicutes as in cell-walled bacteria, a number of fundamentally different substrate uptake systems operate. Transport may be brought about via the generation of a proton-motive force. A membrane-bound ATPase has been identified which functions as an electrogenic proton pump (Linker and Wilson, 1985). A PEP:PTS for which glucose and/or methyl-D-glucoside (α-MG) are substrates, has been demonstrated in fermentative mycoplasmas and spiroplasmas (Cirillo, 1979). However PEP:PTS activity is apparently absent in non-fermentative mycoplasmas and *A. laidlawii* (Cirillo, 1979) and *U. urealyticum* (Cocks *et al.*, 1985).

Tangney *et al.* (1992) reported that maltose might be transported directly by a proton motive force (PMF)-dependent transport system in bacteria. In log phase cells, there are two distinct glucose transport systems; one a PTS and the other a non-PTS transport system. However, in stationary phase cells, only the glucose PTS is active. In enteric bacteria, the inhibition of non-PTS transport system by glucose has been shown to involve a direct interaction between the enzyme IIA component of the glucose PTS and the target non-PTS transport mechanism (Saier, 1989). Glycerol transport, which is via a non-PTS mechanism, is regulated by the glucose PTS in *B. subtilis*. Genomic or enzymatic evidence of amino acid transport or synthesis in mollicutes is limited. The apparent absence of genes for the synthesis of amino acids suggested that there would be a large number of transport systems but such expectation in *M. genitalium* and *M. pneumoniae* were not confirmed by annotation, although histidine, glutamine, oligopeptide and a general amino acid permease transporter were annotated (Fraser *et al.*, 1995, Himmelreich *et al.*, 1996). Paulsen *et al.* (2000) reported the identification of two or three secondary transporters of the amino acid-polyamine-organo cation family (APC) in *M. genitalium* and *M. pneumoniae*. They also identified the presence of MPS and ABC transport families. Convincing identification by putative annotation or expression of a nucleic acid precursor transporter in *M. genitalium*, *M. pneumoniae* and *U. urealyticum* is limited however, they did not identify any specific nucleoside or nucleobase transporter of the 20 or 22 total identified in *M. genitalium* and *M. pneumoniae* respectively. Further, other than components of the PTS-system, proteomic analysis has not revealed any proteins ascribed to purine-pyrimidine transporter (Wasinger *et al.*, 2000). It is assumed that the oligonucleotide and nucleic acids in media are processed by mollicute nucleases that are

surface bound, transported or released from injured or dying cells. The nuclease action produces smaller transportable units that can be assimilated by growing cells (Minon *et al.*, 1993).

1.17 Identification of mollicutes, assignment of families, genera and species

Identification and classification of members of the class *Mollicutes* have been based on phenotypic characteristics, such as the ability to produce acid from glucose, a requirement of urea for growth, and utilisation or hydrolysis of arginine, on serological associations, and on rRNA sequences. The members of the class *Mollicutes* have undergone a remarkable series of genome reductions, as determined by rRNA analysis (Rogers *et al.*, 1985; Weisburg *et al.*, 1989). Major characters of the mollicutes are the absence of cell wall, ability to pass through filter, pleomorphism, resistance to penicillin and its analogues, and typical fried egg colonies (Freundt and Razin, 1984). Acholeplasmas, mesoplasmas, asteroplasmas and some spiroplasmas are distinguished from other mollicutes by lack of sterol requirement and can grow in serum-free media. Growth inhibition tests by digitonin are useful for the primary assessment of sterol requirement (Freundt *et al.*, 1973). Acholeplasmas may also be distinguished from mycoplasmas by their sensitivity to nisin and ability to reduce benzyl viologen (Abu-Amero *et al.*, 1996). The determination of order and family within the class *Mollicutes* are sterol requirement, cellular morphology, optimum growth temperature, the use of UGA or UGG as the codon for tryptophan, genome size and DNA base (G + C mol %) composition, the atmospheric requirement and the ability to hydrolyse urea. Ureaplasma are differentiated by their ability to hydrolyse urea.

The identification of species is generally determined by serological or molecular techniques. Serological tests for the identification of mollicutes are: direct and indirect fluorescence antibody tests (Gardella and DelGuidice, 1983); agar precipitation and immunoelectrophoretic methods (Kenny, 1983); enzyme linked immunosorbent assay (ELISA, Ball and Finlay, 1998); haemaglutination (Jones and Wood, 1988); and complement fixation test (Taylor-Robinson *et al.*, 1966). Serological tests are limited because of serological cross-reactions, for example *M. agalactiae* and *M. bovis* are serologically cross-reactive (Askaa and Ernø, 1976). The members of the '*M. mycoides*

cluster' exhibit significant cross-reactivity (Rodriguez *et al.*, 1996). Additionally, Christiansen *et al.* (1987) reported that mycoplasmas characterised on a serological basis showed substantial genetic differences, for example *M. hominis* the G + C content varies from 27-34 %.

To overcome the problems of cross reactivity, molecular techniques are now widely used to identify microorganisms. Polyacrylamide gel electrophoresis of total protein gives species-species protein patterns (Costas *et al.*, 1987). DNA probes have been developed for the identification of a variety of human, animal, and avian mycoplasmas including *M. agalactiae*, *M. bovis* (Dedieu *et al.*, 1995; Tola *et al.*, 1996), *M. mycoides* cluster, *M. genitalium*, *M. gallisepticum*, *M. pneumoniae* and *M. synoviae* (Taylor *et al.*, 1992; Razin, 1994). The sensitivity of mycoplasma detection by DNA probes ranges between 10^3 and 10^6 cfu. ml^{-1}, a level that may not be suffiently high for general use in clinical laboratories (Razin, 1994). Restriction fragment length polymorphism (RFLP) analysis of DNA has been used to characterise genomes of *M. bovis* and *M. bovigenitalium* (Hotzel *et al.*, 1990). Recently, amplified fragment length polymorphism analysis (AFLP) has been used to assess the genomic diversity among Danish field strains of *M. hyopneumoniae* (Kokotovic *et al.*, 2002). The 16S rRNA genes of mycoplasmas, collected in eight genera, have been the subjects of study mainly for the purpose of taxonomy (Wiesburg *et al.*, 1989).

Athough DNA probes and PCR procedures may enable early diagnosis of infection because they do not always depend on culture, they do require highly skilled technical staff and sophisticated and expensive instruments. Leach *et al.* (1989) reported PAGE of total cell protein fails to distinguish *M. mycoides* subsp. *capri* from *M. mycoides* LC. The use of probes for hybridisation may result in non-specific hybridization giving misleading results; PCR may require lengthy optimisation and for closely related species it may be difficult to design specific primers and in clinical samples, the sensitivity of the PCR detection may be relatively low (Karch *et al.*, 1995). Therefore there is a need for novel methods for the detection and identification of mollicutes from clinical samples.

1.18 Aims of the project

The major aims of the project were to determine the patterns, kinetics and regulation of energy substrate metabolism within members of non-fermentative and non-arginine hydrolysing mycoplasmas including; *M. agalactiae*, *M. bovis*, *M. bovigenitalium* and *M.* ovine serogroup 11. All these four species cause of economically important diseases in animals. Relatively little is known of the metabolism of these organisms, and only the reference strains had been studied previously. The field isolates of *M.* ovine serogroup 11, a cause of infertility in sheep, were isolated in the UK for the first time (Nicholas *et al.*, 1999) and isolates of this organism were also included in the study.

It was envisaged that the information obtained in the biochemical study (Chapter 3, 4) would improve understanding of the potential roles of substrate metabolism and toxic product formation in pathogenicity. Such information is of potential value in epidemiological studies and understanding of the nutritional requirements of these in the non-fermentative and non-arginine hydrolysing mycoplasmas which might be applied to the development of improved culture media. Additionally, if consistent biochemical differences amongst members were found, it might also be used to develop rapid biochemical tests aiding identification. The application of these techniques to the routine identification of mycoplasmas is limited by the necessity for dedicated equipment, which is generally not available in laboratories. During the course of the study, some strains of *M. bovis* that produced high levels of H_2O_2 (a potential pathogenicity factor) were investigated. A study was also conducted, to determine whether *in vitro* passage cause decrease in H_2O_2 which could be valuable for the identification of vaccine candidates.

The esterase (lipase) activity of mycoplasmas was also investigated using chromogenic substrates. It is possible that the esterase/lipase activity detected might be associated with pathogenicity and could be used to differentiate on the basis of lipolytic activity of mycoplasmas. In preliminary experiments, conditions for the qualitative and quantitative lipase test were optimised. SLPA-substrates were used and a rapid qualitative and quantitative assay system was developed. An attempt was also made to detect lipolytic activity on gels. Field strains of the *M. bovis* were characterised using restricted fragment length polymorphism (RFLP), pulsed field gel electrophoresis (PFGE), restriction

endonuclease (RE), immunoblotting and SDS-PAGE. These studies were undertaken to show whether biochemical similarity could be correlated with antigenic and genetic similarity.

Chapter 2

2. Materials and methods

2.1 Organisms used in the study

The *Mycoplasma* type and field strains used in the study were obtained from different sources as shown in Table 2.1. All mycoplasmas were obtained as freeze-dried or broth cultures and upon receipt, cultures were grown in broth medium. All cultures were stored and maintained at –85°C for further studies.

2.2 Growth of organisms

Mycoplasma strains were grown in plastic screw-capped test tubes (Sterilin, Stone, Staffs, UK) having broth medium (7 ml) with initial pH 7.6 and inoculated with 0.5 ml of a –85°C stored culture thawed at 37°C or 0.1 ml subculture. The cultures were incubated statically at 37°C for 24-120 hours depending upon the growth characteristics of the organism; 0.1 ml quantities of these cultures were transferred to fresh medium and similarly incubated. The media used for the growth of organisms are described in Section 2.3. Filter-sterilised (0.2 μm pore size, Gelman, USA) sodium pyruvate (2g/l) was used as energy substrate for the growth of *Mycoplasma agalactiae*, *Mycoplasma bovis*, *Mycoplasma* ovine serogroup 11, *Mycoplasma bovigenitalium* and *Mycoplasma gallinarum*. The grown cultures were stored in 1.5 ml freezing vials (Sigma, Poole, UK) at –85°C for future experiments.

2.3 Growth media

Different media were used for the growth of mycoplasmas according to their growth requirements. All media were stored at 4°C.

2.3.1 Proteose peptone medium

This medium was used for the growth of *M. alkalescens, M. felis and M. gallinarum.* The composition of the medium was (g/l): proteose peptone, 17; neutralised liver digest,

2.5; Na_2HPO_4, 2.5; NaCl, 5; glycerol, 5; sodium pyruvate, 2; yeast extract, 5 and glucose, 5. These components of the medium except pig serum or bovine calf serum (Gibco, Life Technologies, Paisley, UK) 200 ml and sodium pyruvate were added to distilled water (800 ml), the pH was adjusted to 7.6 by adding 10 M NaOH then autoclaved at 121°C for 15 mins. Medium was cooled to room temperature then pyruvate (filter sterilised) and serum was added to the medium and was stored at 4 °C.

2.3.2 PRM medium

All mycoplasmas except where stated were grown in PRM medium (Rice *et al.*, 1999) containing (g/l): special peptone L-72, 20; yeast extract, 5; sodium pyruvate, 2; glucose, 5 g; glycerol, 5; HEPES, 9; fresh yeast extract 100 ml, and heat inactivated porcine serum (Gibco, Life Technologies, UK), 100 ml. The pH was adjusted to 7.6 by adding 10 M NaOH and medium was autoclaved at 121°C for 15 mins. Sodium pyruvate (filter sterilised), serum and fresh yeast extract (Section 2.5) were added to the medium when it was cooled.

2.3.3 Eaton's medium

This medium was used for the growth of *Mycoplasma agalactiae, Mycoplasma bovis, Mycoplasma canis* and *Mycoplasma bovirhinis.* It consisted of 21g PPLO broth base (Difco, Molesey, UK; without crystal violet), which was added to 700 ml distilled water and was autoclaved at 121 °C for 15 mins. The medium was cooled to room temperature and fresh yeast extract (Section 2.5), (100 ml), inactivated horse serum (200 ml), glucose 10 g, 0.5 ml penicillin (200,000 IU ml^{-1}), 0.02 g DNA and phenol 0.2% (v/v) were added. The pH of the medium was adjusted by adding 10 M NaOH and was sterilised by membrane filtration (Gelman, pore size, 0.45 mm).

2.3.4 SP4 medium

This is enriched medium and was initially devised for the isolation of spiroplasmas. Modified SP4 medium (Tully and Whitcomb, 1983) was used for the growth of *Mycoplasma fermentans, Mycoplasma penetrans, Mycoplasma imitans, Mycoplasma felis* and *Mycoplasma gallisepticum.*

This medium consisted of:

Part 1 (g/l):

Mycoplasma broth base, 3.33; proteose peptone, 5.33; tryptose, 10; HEPES, 18. All these components were dissolved in distilled water (657 ml) and the pH was adjusted to 7.6 with 10 M NaOH. It was sterilised by autoclaving at 121°C for 15 mins and was allowed to cool to room temperature.

Part 2:

Yeast extract, 2% (w/v) 100 ml/l; CMRL, 50 ml/l; fresh yeast extract 25% (w/v), 35 ml/l; porcine or bovine calf serum (Gibco, Life Technologies, UK), 167ml/l, glutamine, 4.5% (w/v), 13ml/l; glucose 50% (w/v), 10 ml/l.

The glucose, yeast extract and fresh yeast extract were prepared and autoclaved at 121 ° C for 15 mins separately. Glutamine was filter sterilised (Gelman, 0.22 μm pore size). Porcine and bovine calf serum was inactivated at 56°C in a water bath for 1hour. For non-fermentative and non-arginine hydrolysing mycoplasmas 0.2% (w/v) sodium pyruvate was added. Part 2 components were added to part 1 and the complete medium was stored at 4°C.

2.4 Blood agar medium.

This medium consisted of blood agar base No 2 which was dissolved in distilled water (40 g/l) and the pH was adjusted to 7.6 with 1 M NaOH. After autoclaving (121 °C for 15 mins) the molten agar was cooled to 50°C and inactivated porcine or bovine calf serum (Gibco, Life Technologies, UK) was added to a final concentration of 20% (v/v) by gently rotating the medium and the plates (60 mm diameter) were poured immediately. Petri plates were left at room temperature until dried and were stored at 4°C.

Table 2.1 Organisms used in the study

***Mycoplasma* species**	**Geographical origin**	**Site of isolation**	**Host**
***Mycoplasma agalactiae* NCTC 10123**	Not known[a]	Not known	Ovine
480/79	France[b]	Not known	Ovine
2495/87	France[b]	Not known	Ovine
2123/91	Sicily, Italy[c]	Ocular discharge	Ovine
723/93	Sicily, Italy[c]	Lung	Ovine
1070/93	Palermo, Italy[c]	Milk	Ovine
499/93	Trapani, Italy[c]	Milk	Ovine
1209/93	Sicily, Italy[c]	Vaginal swab	Ovine
29/93	Messina, Italy[c]	Milk	Ovine
471/93	Sicily, Italy[c]	Lung	Ovine
101/94	Sicily, Italy[c]	Milk	Ovine
453/94	Sicily, Italy[c]	Lung	Ovine
730/97	Sicily, Italy[c]	Nasal discharge	Caprine
314/97	Sicily, Italy[c]	Lung	Ovine
1536/98	Caltanissetta, Italy[c]	Milk	Ovine
1896/98	Enna, Italy[c]	Milk	Ovine
432/98	Enna, Italy[c]	Milk	Ovine
2245/99	Messina, Italy[c]	Milk	Caprine
4400/99	Agrigento, Italy[c]	Milk	Ovine
LF/00	Palermo, Italy[c]	Joint fluid	Ovine
4a	Las Palmas, Spain[d]	Udder	Ovine
10a	Las Palmas, Spain[d]	Udder	Ovine
11b	Las Palmas, Spain[d]	Udder	Ovine
6gb	Las Palmas, Spain[d]	Udder	Ovine
Vizcaya 3328(3)	Las Palmas, Spain[d]	Vagina	Ovine
Veterinarialeche 18	Las Palmas, Spain[d]	Milk	Ovine

Table 2.1 Organisms used in the study (continued)

***Mycoplasma* species**	**Geographical origin**	**Site of isolation**	**Host**
***M. bovis* NCTC 10131**	USA[a]	Udder	Bovine
79B96	Thirsk, UK[e]	Lung	Bovine
81B96	Thirsk, UK[e]	Lung	Bovine
82B96	Thirsk, UK[e]	Lung	Bovine
119B96	Thirsk, UK[e]	Lung	Bovine
193B96	Bristol, UK[e]	Not known	Bovine
233B96	Winchester, UK[e]	Lung	Bovine
10B97	Thirsk, UK[e]	Lung	Bovine
33B97	St. Edmunds, UK[e]	Lung	Bovine
139B97	Penrith, UK[e]	Not known	Bovine
67M98	Penrith, UK[e]	Not known	Human
135B99	Winchester, UK[e]	Lung	Bovine
136B99	St. Edmunds UK[e]	Lung	Bovine
137B99	Penrith, UK[e]	Lung	Bovine
139B99	Newcastle, UK[e]	Lung	Bovine
142B99	Winchester, UK[e]	Not known	Bovine
156B99	Thirsk, UK[e]	Lung	Bovine
5B00	Shrewsbury, UK[e]	Lung	Bovine
7B00	Thirsk, UK[e]	Lung	Bovine
8B00	Thirsk, UK[e]	Lung	Bovine
10B00	Penrith, UK[e]	Bronchi	Bovine
12B00	Luddington, UK[e]	Lung	Bovine
20B00	Preston, UK[e]	Lung	Bovine
27B00	Aberystwyth, UK[e]	Not known	Bovine
51B00	Carmarthen, UK[e]	Milk	Bovine
55B00	Newcastle, UK[e]	Lung	Bovine
56B00	Thirsk, UK[e]	Lung	Bovine

Table 2.1 Organisms used in the study (continued)

***Mycoplasma* species**	**Geographical origin**	**Site of isolation**	**Host**
***M.* ovine serogroup11, 2D**	Not known[a]	Not known	Ovine
48SR98	Preston, UK[e]	Not known	Ovine
50SR98	Preston, UK[e]	Not known	Ovine
52SR98	Preston, UK[e]	Not known	Ovine
3SR99	Evesham, UK[e]	Vaginal swab	Ovine
47SR99	Preston, UK[e]	Vaginal swab	Ovine
48SR99	Preston, UK[e]	Vaginal swab	Ovine
52SR99	Preston, UK[e]	Vaginal swab	Ovine
95SR99	Preston, UK[e]	Preputial swab	Ovine
96SR99	Preston, UK[e]	Semen	Ovine
126SR99	Preston, UK[e]	Vaginal swab	Ovine
129SR99	Preston, UK[e]	Sheath swab	Ovine
***Mycoplasma bovigenitalium* NCTC 10122**	Not known[a]	Genital tract	Bovine
434/81	Germany[f]	Bull semen	Bovine
398/87	Germany[f]	Ram semen	Ovine
57B00	Bristol, UK[e]	Vaginal swab	Bovine
***Mycoplasma bovirhinis* NCTC 10118**	Not known[b]	Respiratory tract	Bovine
1B00	Penrith, UK[e]	Lung	Bovine
11B00	Penrith, UK[e]	Lung	Bovine
15B00	Thirsk, UK[e]	Lung	Bovine
16B00	Starcross, UK[e]	Lung	Bovine
22B00	St. Edmunds UK[e]	Lung	Bovine
29B00	Shrewsbury, UK[e]	Lung	Bovine

Table 2.1 Organisms used in the study (continued)

***Mycoplasma* species**	**Geographical origin**	**Site of isolation**	**Host**
***M. mycoides* SC NCTC 10114**	Not known[a]	Lungs	Bovine
V5	Australia[b]	Vaccine strain	Bovine
130/20P	Italy[b]	Lungs	Bovine
Clone 1-14	Portugal[b]	Lungs	Bovine
SH9	Kavongo, Namibia[g]	Lung	Bovine
Oremit	Kenya[b]	Not known	Caprine
Segonia	Not known[e]	Not known	Bovine
IMVT	Not known[e]	Not known	Bovine
***Mycoplasma mycoides* LC**	Australia[b]	Peritoneal exudate	Caprine
***Mycoplasma mycoides* subsp. *capri* 10137**	Not known[b]	Lungs	Caprine
BQT	Turkey[b]	Not known	Caprine
Pendik	Turkey[b]	Not known	Caprine
***Mycoplasma canis* NCTC 10146**	Not known[a]	Throat	Canine
17B95	Thirsk, UK[e]	Nasal swab	Bovine
113B97	Bristol, UK[e]	Lung	Bovine
171B97	Winchester, UK[e]	Nasal swab	Bovine
219B97	Penrith, UK[e]	Not known	Bovine
85B98	Penrith, UK[e]	Not known	Bovine
92B98	Aberystwyth, UK[e]	Not known	Bovine
2B00	Penrith, UK[e]	Lung	Bovine
93B98	Aberystwyth, UK[e]	Not known	Bovine
96B98	Winchester, UK[e]	Lung	Bovine

Table 2.1 Organisms used in the study (continued)

***Mycoplasma* species**	**Geographical origin**	**Site of isolation**	**Host**
9B00	Penrith, UK[e]	Lung	Bovine
46B00	Winchester, UK[e]	Lung	Bovine
***Mycoplasma dispar* NCTC 10125**	Not known[a]	Lung	Bovine
18B00	Bristol, UK[e]	Lung	Bovine
25B00	Bristol, UK[e]	Lung	Bovine
***Mycoplasma fermentans* NCTC 10117**	Not known[a]	Not known	Human
MF Sheep	UK[b]	Genital tract	Sheep
10E	UK[b]	Leukaemic bone marrow	Human
262	UK[b]	Leukaemic bone marrow	Human
2059	UK[b]	Cell lines	Human
28AC	UK[b]	Cell lines	Human
Incognitus strain	UK[b]	Kaposis sarcoma	Human
***Mycoplasma pullorum* 50SR99**	UK[e]	Not known	Avian
***Mycoplasma alkalescens* NCTC 10135**	Not known[a]	Nasal cavity	Bovine
***Mycoplasma penetrans* ATCC 55252**	Not known[a]	Not known	Human
***Mycoplasma felis* NCTC 10160**	UK[f]	Not Known	Feline

Table 2.1 Organisms used in the study (continued)

***Mycoplasma* species**	**Geographical origin**	**Site of isolation**	**Host**
***Mycoplasma gallinarum* NCTC 10120**	Not known[a]	Respiratory tract	Avian
***Mycoplasma imitans* NCTC 11733**	UK[b]	Turbinate	Avian
***Mycoplasma caviae* NCTC 10126**	Not known[b]	Not known	Guinea pig
***Mycoplasma edwardii* NCTC 10132**	Not known[a]	Throat	Canine
***Mycoplasma columbinum* 10178**	Japan[b]	Trachea	Avian
***Mycoplasma columbinasale* 10184**	Not known[a]	Turbinate	Avian
***Mycoplasma putrefaciens* 10155**	Not known[a]	Not known	Caprine
***Mycoplasma salivarium* 10113**	Not known[a]	Saliva	Human
***Mycoplasma verecundum*10145**	Not known[b]	Eye	Bovine
***Acholeplasma axanthum* 10138**	UK[a]	Tissue culture	Murine
***Acholeplasma laidlawii* 10116**	Not known[b]	Not known	Bovine
***Acholeplasma oculi* 10150**	Not known[b]	Eye	Caprine

Cultures were kindly supplied by:

(a) Purchased from Public Health Laboratory, Colindale Avenue, London, UK. (b) –85° C culture storage, King's College London, Department of Life Sciences, 150 Stamford Street, SE1 9NN, London, UK. (c) Instituto Zooprofilattico Sperimentale della Sicilia " A Mirri" Via R Dicillo, 4 90129 Palermo, Italy. (d) Dr Jose B Poveda, Universidad de Las Palmas de Gran Canaria, Facultad de Veterinaria, Las Palmas, Espana. (e) Dr Robin Nicholas, Veterinary Laboratories Agency, New Haw, Addlestone, Surrey KT 15 3NB, UK. (f) Dr K Sachse, Federal Institute for Health Protection of Consumers and Veterinary Medicine (BgVV), Division 4, Naumburger Str.96a 07743 Jena, Germany. (g) Dr John B March, Moredun Research Institute, Penicuik Pentland Science Park, Bush Loon, EH 26 OPZ, Penicuik, UK.

This medium was used for the total viable count determination, to check the purity of the culture and purification of specific isolates by cloning three times according to the method of Tully (1983).

2.5 Fresh yeast extract

Fresh yeast extract 25% (w/v) was prepared as described by Freundt (1983), in which dried baker yeast extract (250g; Allinson, West-mill Food Ltd., UK) was mixed with 1litre-distilled water, heated with stirring for 15 mins and subsequently cooled.

The solution was centrifuged at 11300 x g (Beckman, Bucks, UK) for 30 mins at room temperature (20°C). The supernatant was separated and filtered with Whatman No 1 filter paper. The pH of the filtrate was adjusted to 7.6 with 10 M NaOH and the filtrate was autoclaved at 121°C for 15 mins. After cooling it was stored at –20°C.

2.6 Suspension media

2.6.1 Ringer HEPES buffer (RH)

Ringer HEPES buffer was used for washing and suspension of organisms for substrate oxidation procedures and viable counts. HEPES (9 g) and 1 tablet of Ringer's (¼ strength) were dissolved in 500 ml distilled water and the pH was adjusted to 7.6 with 10 M NaOH. The solution was autoclaved at 121°C for 15 mins. Catalase (final concentration 0.04%, w/v) was filter sterilised (0.2 μm) and added to the RH buffer for washing and resuspension of cells before experiments. It was omitted during hydrogen peroxide production experiments. The filter-sterilised catalase solution was stored at –20 °C in 1.5 ml Eppendorf tubes.

2.6.2 Normal saline solution

Sodium chloride (0.85% w/v) adjusted to pH 7.6 by addition of 0.1M NaOH was sterilised at 121°C for 15 mins. Normal saline was used for the washing and resuspension of cells for pH monitoring.

2.6.3 Salt 1 solution

This solution was used to wash and resuspend the cells for protein concentration determination. Salt 1 solution was made by adding g/l: Na_2HPO_4, 32.4; K_2HPO_4, 7.6; NH_4Cl, 4 and NaCl, 6. The pH of the solution was adjusted to 7.6 with 10 M NaOH and autoclaved at 121°C for 15 mins.

2.7 Preparation of cell suspension

Cell suspensions were prepared by dispensing culture in sterilised Eppendorf tubes and centrifuged at 13,000 x g in a MSE micro-centaur centrifuge for 4 mins. The supernatant was discarded and the pellet obtained was washed twice with RH buffer with catalase. The cells were resuspended in 2 ml RH buffer and the optical density (OD) of washed cells was measured at 540 nm in a 1cm cuvette in a Gallenkamp spectrophotometer. The OD of cell suspension was adjusted to 1.0, which was approximately equivalent to 250 μg protein ml^{-1}. The cells were harvested towards the end of the exponential growth phase. The time for the preparation of cell suspension was kept to a minimum (<30 mins) because metabolic activity declines even if cells are kept at 4°C.

2.8 Measurement of optical density (OD)

The growth of mycoplasmas was monitored by measuring the OD of the broth cultures. The OD was measured using 1cm disposable cuvettes in a Gallenkamp visi-spectrophotometer at 540 nm (Miles, 1992a).

2.9 Viable count determination

Serial ten-fold dilutions of cell suspensions were made in Ringer-HEPES buffer in 1.5ml Eppendorf tubes and 5μl of appropriate dilutions were inoculated on to agar plates and were kept at room temperature until dried. The plates were incubated at 37°C for 24-120 hours or until the development of colonies. Colonies were counted by using Kyowa plate microscope under low power and total viable count was expressed as colony forming units (cfu) per ml (Rodwell and Whitcomb, 1983).

The 95% confidence limit of the number of colonies was calculated by the formula $x \pm 1.96\sqrt{x}$ (Meynell and Meynell, 1970) where x = total number of colonies counted and 95% confidence counts were within ± 10 % of the values obtained.

2.10 Protein concentration determination

The protein concentration of the cells was determined by the Bradford (1976) and Markwell *et al.* (1978) methods. Markwell's method consisted of Reagent A, g/l: Na_2CO_3, 20; NaOH, 4; sodium tartarate, 1.6; and sodium dodecyl sulphate, 10. Reagent B: $CuSO_4.5H_2O$ 40, and Reagent C, was made by adding 1 part of reagent B to 100 parts of reagent A. Sample (1 ml) was added to solution C (3 ml), mixed and was incubated at room temperature for 1 hour, then 0.3 ml Folin-Ciocalteu reagent (50% v/v) was added. The mixture was again vortexed and was left at room temperature for 45 mins. The OD was then measured at 660 nm. The samples were harvested by centrifugation at 13,000 x g for 4 mins using a MSE micro-centaur centrifuge. The pellets were washed twice in salt 1 solution and were resuspended in salt 1 solution. The protein concentration was determined from standard curves of bovine serum albumin (Appendix 1), which were prepared fresh during each assay.

2.11 Determination of substrate utilisation

2.11.1 Measurement of oxygen uptake and preparation of oxygen electrode

The pattern of substrate oxidation was determined from changes in dissolved oxygen tension (DOT) measured using a Clark-type oxygen electrode (Rank Brothers, Bottisham, Cambridge, UK) linked to a chart recorder (Gould-BS272). The electrodes (Figure 2.1) were mounted beneath the reaction vessel and were separated from the vessel contents by a semipermeable Teflon membrane which allowed the diffusion of O_2, and contact between the two electrodes was maintained by lens tissue soaked with the saturated KCl. A small hole was made in the tissue, which was placed over the platinum electrode to maintain the contact between the membrane and the electrode. The electrodes were rinsed in distilled water and dried. The reaction vessel was thoroughly washed and cleaned with sterile distilled water and was assembled. An 'O' ring was used to fix the membrane. The contents of the vessel were magnetically stirred and the temperature of the reaction vessel was kept at 37°C by a circulating water pump. The polarising voltage was adjusted to 0.65 volts and the current output from the oxygen

electrode which was proportional to oxygen tension and was recorded on a chart recorder. After complete washing of the electrodes, the system was calibrated with distilled water (1 ml).

The output from the electrode was adjusted to give approximately 95% deflection on the chart recorder, which was equivalent to 210 nmol/ml of O_2 at 37°C. Zero current was confirmed by adding a few crystals of sodium dithionate, which show reduction in DOT within a few seconds. The reaction vessel was completely washed with distilled water, which was then replaced by cell suspension (1 ml).

Cells suspension containing approximately 10^9 cfu/ml, was saturated with air and then isolated from air with a plug with a fine central hole to enable the addition of test substrates. The plug was adjusted in such a way that the meniscus of the cell suspension was within centre of the hole. When stable DOT was obtained test substrates were added through the central pore of the plug using a Hamilton microsyringe. Oxygen uptake was detected from the reduction in DOT and values for the saturation constant (K_s) and maximum velocity (V_{max}) of substrate utilisation were determined (Miles and Agbanyim, 1998).

The system was reoxygenated as required by raising the plug and allowing air to enter the vessel. The concentration of the substrates was: organic acids and isopropanol (100 μM), ethanol, acetaldehyde and propanol (100 μM, 10 mM), sugars (25 μM to 2.5 mM). If the lowest concentration of the substrate was not oxidised then the highest concentration was added.

2.11.2 Determination of NADH oxidase, glycerophosphate oxidase and glycerol kinase activities.

These activities were measured by using an oxygen electrode. Cells were prepared in the same way as Section 2.7 except that catalase was omitted. The oxygen electrode was calibrated and cell suspension transferred to the electrode chamber. The cell suspension was saturated with oxygen and then isolated from air. Triton X-100 (5 μl) was added to lyse the cell suspension. Freshly prepared nicotinamide adenine dinucleotide reduced form (NADH) and α-glycerophosphate (GP) was added to the cell

suspension and any decrease in DOT was monitored on the chart recorder. Glycerol kinase activity was determined from the rate of GP oxidation by lysed cells to which glycerol (4 µl), ATP (4 µl). $MgSO_4$ (4 µl) and GP oxidase (0.5 units) were added and the activity was shown as decrease in DOT.

2.11.3 Hydrogen peroxide production by lysed and whole cells

Hydrogen peroxide (H_2O_2) was measured in lysed and whole cells from increase in DOT. Cell culture was harvested and resuspended in RH buffer (Section 2.7), except the suspension medium did not contain catalase. This was done to enable detection of any H_2O_2 produced during NADH and GP oxidation, which was determined by the addition of catalase (Miles *et al.*, 1991). Cell suspension (1 ml) was added to the oxygen electrode vessel, and a stable chart recorder output was obtained. The cells were then lysed with Triton X-100 (5 µl.). Freshly prepared NADH (2 mM, 10 µl) and GP (2.5 mM, 10 µl) were then added and oxidation was followed by decrease in DOT. When oxygen uptake ceased catalase (5 µl, 40 mg ml^{-1}) was added and any increase in DOT was noted. In separate control experiments, with suspension medium without cells, it was shown that an increase in DOT was proportional to added H_2O_2 on addition of catalase.

2.12 Metabolism monitored by pH change

The pH change was monitored with a pH electrode system (Russell, PHM, 110-070N) connected to a water-jacketed reaction vessel to maintain the temperature at 37°C. Washed cell suspensions in normal saline (10^9cfu/ml) with catalase were prepared (Section 2.7). Cell suspension (2 ml) was placed in the electrode chamber and kept in contact with air. The pH electrodes were fixed by dipping in the cell suspension, which were linked to a chart recorder (Graphic 1002, Lloyd Instruments, Hampshire, UK) and waited until a stable neutral pH was obtained. Substrates were added using a Hamilton microsyringe and any change in pH was recorded on the chart recorder. Values for the saturation constant (K_s) and maximum velocity (V_{max}) of substrate utilisation were determined (Miles and Agbanyim, 1998).

2.13 Qualitative and quantitative detection of lipolytic activity in mycoplasmas

2.13.1 Preparation of chromogenic substrates

The SLPA esters of acetate, propionate, butyrate, hexanoate, octanoate and decanoate were dissolved in 50 % (v/v) methanol in Ringer-HEPES buffer (pH 7.6) and were used immediately.

2.13.2 Qualitative detection of lipase activity

Lipase activity was determined using novel chromogenic substrates (Figure 2.2). SLPA ester solutions (400 µl) were applied to colonies and plates incubated at 37°C for the development of colour. The mycoplasma colonies were observed under the microscope (Kyowa) every 15 mins. The development of red colour within limited time (<60 mins) was recorded as positive. Ester hydrolysis was indicated by the appearance of a bright red colour.

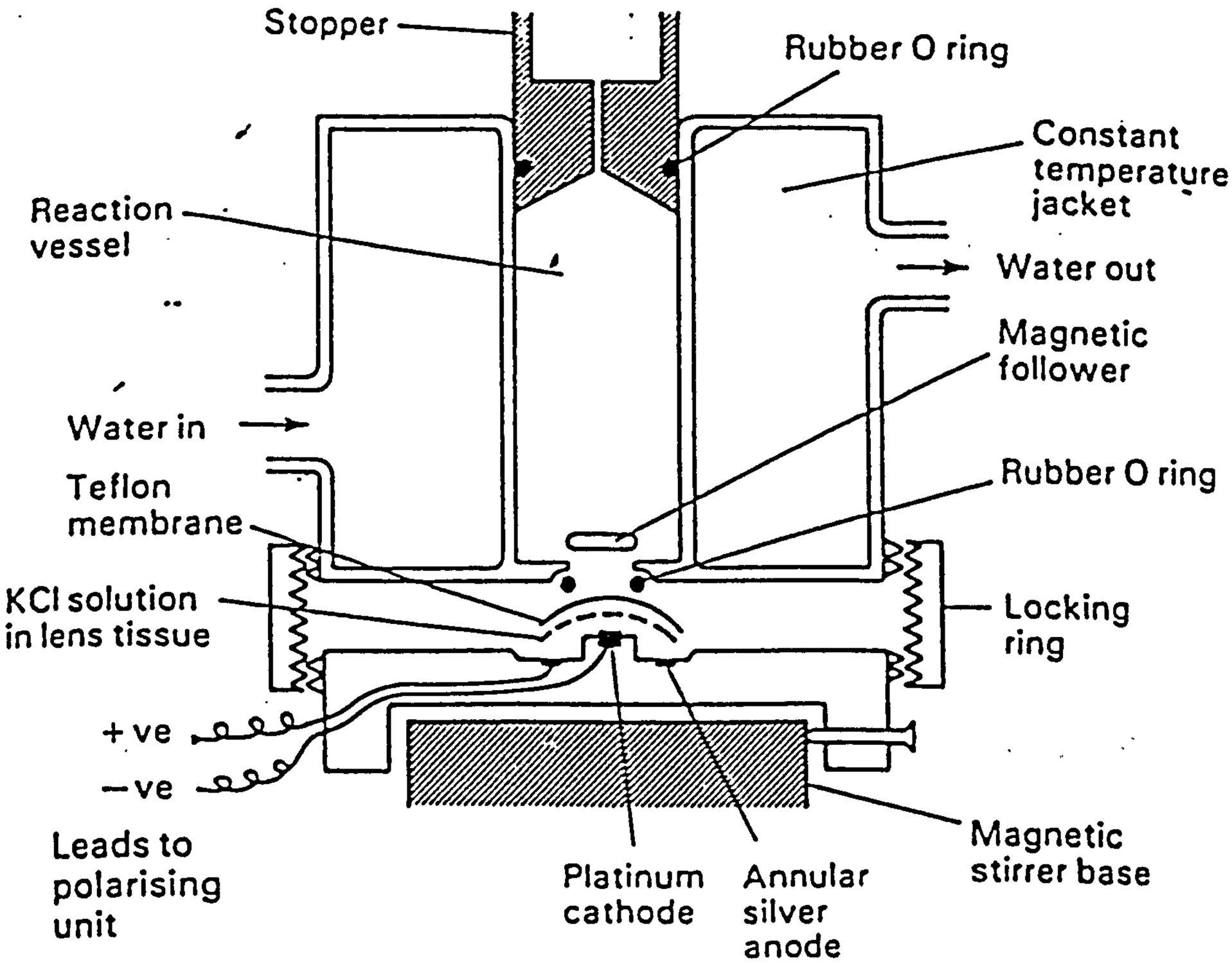
2.13.3 Preparation of *Mycoplasma bovis* cytoplasmic and membrane fractions for the quantitative analysis of the lipolytic activity.

Mycoplasma bovis cells were harvested by centrifugation twice at 5200 x g for 30 mins at 4°C and resuspended in Ringer-HEPES buffer (pH 7.6). Washed cells (40 ml) were lysed by sonication (Soni Prep 50, MSE) for 2.5 mins at maximum wattage with the sample held continuously on ice. The probe was cooled for 45 seconds between cycles. The broken cells were then ultracentrifuged at 154,000 x g at 4°C for 2 hours (Beckman L8-80 Multra centrifuge, USA). The pellet was resusupended in buffer and again centrifuged at 154,000 x g at 4°C for a further 30 mins. The supernatant was removed and the pellet was mixed with 15 ml of Ringer-HEPES buffer, which was used immediately or stored at –80 °C.

2.13.4 Lipase assay using spectrophotometric method.

The maximum absorption spectra (λ_{max}) of the SLPA-phenol were investigated by using a scanning spectrophotometer (Appendix 2). A lipase assay was developed using SLPA-octanoate chromogenic substrate (Section 5.3.2) and conditions were optimised for the assay. Absorbance was measured at 595 nm and phenol released was determined with reference to standard curve (Appendix 2).

Figure 2.1 Clark-type oxygen electrode system (Jenkins, 1988)



2.13.5 Lipase assay using fluorimetric method.

The lipase activity was measured using the methods of Roy (1980) and Stead (1984). The substrate used for the fluorimetric assay was 4-methylumbelliferone ester of heptanoate, which was made up to the concentration of 16.6 mmol l^{-1} by dissolving in 1.5 ml 2-methoxyethanol. Emulsions of the substrate were further diluted with distilled water to 25 ml

The reaction mixture consisted of 10 µl of cytoplasmic or membrane fraction or whole cells, 100 µl substrate, 855 µl Ringer-HEPES buffer and 45µl 0.1 mmol l^{-1} of $CaCl_2$. The fluorescence was continuously monitored using a luminescence spectrometer (Perkin Elmer LS-5) at an excitation wavelength of 339 nm and emission wavelength of 444 nm. The activity was measured with reference to the standard curve of 4-methylumbelliferone (Appendix 2).

2.14 Detection of lipolytic activity in *M. bovis* on polyacrylamide gels.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed for the detection of lipolytic activity in *M. bovis* using the method of Lincolin *et al.* (1994) and was modified. The samples were not boiled and SDS in the gels, sample buffer was used at different concentrations (0-10 % w/v). SDS-PAGE (12.5 % w/v) was performed at 4°C and at 150 volts for two hours. The gels were soaked in coomassie blue for 30 mins and then were destained for the same time.

After staining and destaining gels, renaturation of the enzyme was carried out. The gels were first rinsed in distilled water, placed in 250 ml 0.1% (v/v) Triton-X100 and were shaken gently 30 mins to wash the SDS from the gels. The gels were rinsed again in distilled water and were subsequently incubated in 100 ml Ringer-HEPES buffer for 30 mins. The gels were incubated with 25 ml 4-methylumbelliferone heptanoate (16.6 mmol l^{-1}) for 30 mins. Enzyme activity was detected under ultra violet (UV) light. The lipase activity was also determined on partial denaturing and native gels. Gels were not stained and destained and the renaturation step was omitted. Gels were run for two hours and were incubated with 4-methylumbelliferone-heptanoate (16.6 mmol l^{-1}) for 30 mins at room temperature (18 °C) were examined under UV light and photographed.

2.15 Preparation of *M. bovis* cells for genetic analysis

All strains of *M. bovis* tested were grown as previously described (2.2) and the cells were harvested in the late exponential phase by centrifugation at 5200 x g for 2 hours at 4°C. The supernatant was removed and the pellet was again spun in the cold Ringer-HEPES buffer and were resuspended in the same buffer and stored at –85°C until use.

2.15.1 Extraction of DNA

All the cell pellets were treated with 3 ml TNE buffer containing 1 % (w/v) sodium dodecyl sulphate (SDS) (0.01 M Tris HCl, pH 8.0, 0.01 M NaCl, and 0.01 M EDTA). Proteinase K (20 µl, 20 mg /ml) was then added to each tube and the samples were kept at 37°C for 2 hours. DNase free RNase A was added at the final concentration of 100 µg/ml and the samples were further incubated for 30 mins.

The phase lock gel (PLG) was spun for 1 min to pellet the gel. Phenol saturated with TE buffer (0.01 M Tris-HCl, pH 7.2; 0.01 M EDTA) in 3 ml quantity was added to all the tubes and was mixed to resuspend. The suspension was centrifuged at 13000 x g for 5 mins. The upper phase was removed into fresh tubes with pelleted PLG and 3 ml of phenol: chloroform: isoamyl alcohol (25:24:1) was added to all the tubes and the contents were mixed by gentle shaking and were again centrifuged for 5 mins.

The clear supernatant (upper phase) was removed into fresh tubes in which 300 µl 3M sodium acetate buffer, pH 7.0 and 8 ml cold (–20°C) absolute alcohol were added. The samples were left at –20°C overnight. The samples were centrifuged at 13000 x g for 5 mins and the supernatant was discarded. The pellets were mixed with 200 µl of cold (–20°C) 70 % alcohol and were again centrifuged at 13,000 x g for 5 mins. The supernatant was discarded and the pellet was dried in air for approximately 10 mins. The DNA was reconstituted by mixing the pellet with 50 µl TE buffer and was stored at –20°C.

2.15.2 DNA quantification

The extracted DNA was run on an agarose gel to check the purity (protein-free DNA) of the DNA. The DNA fragments were separated on a 0.7 % (w/v) agarose gel in 1x TAE (0.01 M Tris-HCl, pH 7.2, 0.01 M EDTA) buffer, which contained ethidium bromide

(0.5 μg/ml). The solution was heated in the microwave for 2-3 mins and on cooling it was poured in the casting tray. TAE buffer 1x was used in the tank. The gel was run at 70 volt until the dye reached the end. After electrophoresis the gel was examined on a transilluminator under UV light and was photographed. The amount of DNA was estimated by making comparisons with the 1kb ladder. The concentration and purity of the DNA were also measured by ultraviolet spectophotometry. The concentration of DNA was estimated from the absorbance at 260 nm and an A_{260} of 1.0 corresponds to 50 μg of double stranded DNA/ml. The purity of the DNA was also checked from the ratio of absorbance at 260 nm to that of 280 nm. A ratio of 1.7-2.0 was considered acceptable (Carle *et al.*, 1983).

2.15.3 Detection of *M. bovis* by PCR

The oligonucleotides for the detection of *M. bovis* are listed in Table 2.2. The primers used of the oligonucleotide primer pair MBOUVRC2-L/R, were designed from the *uvr*C gene sequence. PCR with this primer pair amplified a 1.6 kb fragment (for sequence appendix 3) from 18 *M. bovis* strains (Subramaniam *et al.*, 1998).

PCR was carried out in 50 μl reaction mixtures using a thermal cycler (Perkin-Elmer, New Jersey, USA). The reaction mixture consisted 1μl of 20 pmol of primer 1 and 2, 1mM dNTP, 10x PCR buffer, 2.5 mM $MgCl_2$, 1.25 U of Taq polymerase (Promega). The reaction mixture was made up to 49 μl by the addition of deionised water and 1 μl of DNA was added. The tubes were kept in the thermal cycler for 33 cycles with the parameters 30 seconds at 94°C, 30 seconds at the corresponding annealing temperature 60 °C and extension for 1 min at 72°C. After the completion of PCR the product was analysed by 2 % (w/v) agarose gel electrophoresis and was visualised under a UV transilluminator and photographed.

Table 2.2 Oligonucleotide primers for PCR detection of *M. bovis*

Primer	Nucleotide position*	Sequence
MBOUVRC2-L	362-381	5' TTACGCAAGAGAATGCTTCA -3'
MBOUVRC2-R	1988-1969	5' TAGGAAAGCACCCTATTGAT-3'

* Position according to Genbank accession number AF 003959

2.15.4 Restriction enzymes digestion of PCR product.

The DNA samples (5 µl) in TE buffer were mixed with the restriction enzymes *Asn*1, *Dde*I (10 units μl^{-1} Boehringer) and *Ssp*1 (10 units μl^{-1}) with 1 µl of 10 x restriction buffer. The volume of the reaction mixture was 10µl and the digestion was performed for 1-2 hours at 37°C. The digested DNA with loading buffer (0.25 % (w/v) bromophenol in 40 % (w/v) sucrose) was mixed and loaded onto agarose gel. The 4% Invitrogen gel was run until the dye reached the end of the gel. The gel was examined under UV light and photographed.

2.16 Restriction enzymes digestion of whole genomic DNA.

The DNA samples (1-10 µl in TE buffer) were mixed with the restriction enzymes (10-70 units μl^{-1}) with 1 µl of 10 x restriction buffer. The restriction enzymes used for the digestion of whole genomic DNA were, *Bam*HI, *Bgl*I, *EcoR*I, *Hind* III, *Pst*I and *Sma*I. The volume of the reaction mixture was 10µl or 20µl and the digestion was performed for 2 hours or overnight at 37°C. The digested DNA with loading buffer (0.25% (w/v) bromophenol in 40 % (w/v) sucrose) was mixed and loaded onto 1% (w/v) agarose gel. The gel was run at 70 volt until the dye reached the end of the gel. The gels were also run at 4°C overnight at 30 volt. The gel was examined under UV light and was photographed.

2.17 Comparison of restriction patterns of *M. bovis* by pulsed field gel electrophoresis (PFGE).

Genomic DNA was digested using restriction enzymes, which gave clearly separated DNA fragments with a uniform size distribution. The restriction enzyme used was *Sma*I. *M. bovis* cells were grown as previously described in Section 2.2. Cells were pelleted at 20, 000 x g for 30 mins, washed and resuspended in PBS (0.1 M Na_2HPO_4, NaH_2PO_4; 0.33 M NaCl, pH 7.4).

2.17.1 Preparation of DNA and PFGE analysis

The methods of Tola *et al.* (1999) and Kusiluka *et al.* (2000) were followed for PFGE. Mycoplasma washed cells and equal volumes of low melting point agarose (2 % (w/v) in PBS, pH 7.4) were mixed together and aliquoted. The aliquots of the supension (100

µl) were placed in a mould and allowed to solidify at 4 °C. After solidification blocks were incubated in a lysis solution (1 % (w/v) sarkosyl, 0.5 M EDTA, 10 mM Tris, pH 9.5) supplemented with 2 mg/ml proteinase K for 48 hours at 50 °C. Blocks were washed twice with phenylmethylsulphonyl fluoride (PMSF, 40 µg/ml in TE) for 30 mins at 50 °C. Blocks were again washed with 0.5 M EDTA, pH 8.0 for 30 mins. at room temperature and stored at 4 °C in 0.5 M EDTA, pH 8.0 and were cut into slices (approx. 50 µl).

Agar slices were equilibrated for 15 mins at 4 °C with the respective restriction buffer (500 µl) then the restriction buffer was removed, replaced with 150 µl fresh buffer containing 10-40 enzyme units and 500 µg/ml acetylated bovine serum albumin in TE buffer. Agarose ultra pure running gel (1 % w/v) was prepared in 0.5 x TBE buffer (45 mM Tris, 45 mM boric acid, and 2 mM EDTA, pH 7.6). PFGE was performed at 14 °C in a contour-clamped homogeneous electric field at 6 v cm^{-1} for 20 hours with a 0.4 to 40 seconds pulse ramp time at an included angle of 120 °C using a 200 kbp Lambda ladder (Sigma) as a DNA marker. After completion, the electrophoresis gel was stained with 1 µg/ml ethidium bromide for 15 mins, destained with water for 15 min, visualised under a UV transiluminator and photographed.

2.18 Growth and preparation of *M. bovis* and other species for protein profile and immunoblotting.

2.18.1 Harvesting of organisms

M. bovis and other species tested were grown as in Section 2.2 and the cells were harvested in the late exponential phase by centrifugation at 5200 x g for 1 hour at 4°C. The supernatant was removed and the pellet was again spun in cold 0.01M Tris-HCl buffer and was resuspended in the same buffer and used immediately or stored at –20°C until use.

2.18.2 Preparation of the gels

SDS-PAGE was performed using the method of Laemmeli (1970) with a Bio-Rad Mini Protean II system (Bio-Rad, Hempstead, UK) which was assembled according to the

manufactures instructions. The resolving gel (12.5 %) was prepared by adding acrylamide 4.2 ml, bis-acrylamide 1.69 ml, resolving buffer Tris-HCl (27.23 g Tris base was dissolved in 80 ml distilled water, pH adjusted to 8.8 with 1M HCl and brought to a total volume of 150 ml with distilled water and was stored at 4°C) 2.5 ml, 10 % (w/v) SDS 100 µl, ammonium persulphate (APS) (10 % w/v, freshly prepared) 100 µl and TEMED (NNNN-tetramethylethylenediamine), 5 µl and distilled water 1.6 ml. All the reagents except APS and TEMED were mixed and the solution was degassed for 15 mins. After degassing, the APS and TEMED were added by gentle rotating and the solution was poured (approximately 1cm below the comb teeth) smoothly avoiding air bubbles. Water-saturated isopropanol was overlaid and the separating gel was allowed to polymerise for 45 mins.

The stacking gel (5 % w/v) was prepared by adding acrylamide 0.71 ml, bis-acrylamide 0.3 ml, stacking buffer Tris-HCl (Tris base, 6 g was dissolved in 40 ml distilled water, titrated to pH 6.8 with 1M HCl, then brought to a total volume of 100 ml with distilled water). The buffer was stored at 4°C and 1.36 ml was added to the stacking gel, distilled water 2.62 ml, 10 % APS 100 µl, and TEMED 5µl. All the components of the stacking gel were mixed except APS and TEMED and the solution was degassed for 15 mins. The separating gel was rinsed with distilled water and was dried with filter paper. The stacking gel was poured between the glass plates, a comb was inserted and the gel was allowed to polymerise for 45 mins. The comb was removed gently and the wells were rinsed with distilled water. The electrophoresis equipment was assembled, the polymerised gels were fixed in the instrument and the gel tank was filled with running buffer. This buffer consisted of the following components g/l: Tris base 30.3, Glycine 144.0, SDS 10.0. The buffer was stored at 4°C and 1x buffer was used as a running buffer.

2.18.3 Preparation of the samples and electrophoresis

Samples were mixed with loading buffer (glycerol 2.5 ml, 0.5 M Tris-HCl, pH 6.8, 1.25 ml; SDS 10 % (w/v) 2 ml mixed with 3.55 ml of distilled water, β mercaptoethanol was added prior to use and buffer was kept at room temperature) with the variable volume to get the final protein concentration 5 µg per well or required. The samples along with the loading buffer were boiled for 4 mins and loaded in gels. Novax 200-kDa prestained

marker was used. The samples were run at 150 volt for 1 hour.

2.18.4 Staining and destaining of the gels

The gels were stained with coomassie blue R (0.1% (w/v), coomassie brilliant blue, 10 % (v/v) acetic acid, 40 % (v/v) methanol in distilled water) for 1-2 hours and were destained for the same time with the destaining (30 % (v/v) methanol and 10 %(v/v) glacial acetic acid in distilled water). The gels were photographed using Herolab, Enhanced Analysis system (Wieslock, Germany).

2.19 Immunoblotting method

SDS-PAGE (12 %) gel was run at 70 volt overnight at room temperature (18 °C). The gel was then transferred to a container containing transfer buffer: (SDS 10 % (v/v) solution 7.5ml, Tris-base 48 mM, 11.64 g; glycine 39 mM, 5.86 g; methanol 400 ml and distilled water 1592.5 ml and allowed to equilibrate). The blotting papers and gels were soaked in transfer buffer for 10 mins. The membranes were transferred to the semi–dry blotter, which was rinsed with distilled water before use. The gels and membrane were transferred placing one blotting paper on the bottom, membrane, gel in the middle and one blotting paper was kept on the top of the gel. Air bubbles were avoided. The blotting was done for 90 mins at a constant 15 volt.

After completion of blotting, membranes were stained to check the transfer of the protein. The membranes were covered with Ponceau stain for approximately 1 min and then the dye poured off and the membranes were rinsed with distilled water. The edges of the gel were marked and the membranes were completely washed with phosphate buffered saline (PBS) to remove the stain.

The membranes were then transferred to the blocking solution (which consisted of glycine 1M, 7.5g; chicken egg albumin, 1g and skim milk 5g. All these components were dissolved in 100 ml PBS and blocking solution was prepared fresh each time), were blocked for 1 hour and subsequently washed in 0.1M PBS with 0.1 % (v/v) Tween-20. The membranes were air-dried and kept between the filter paper at room temperature. The membranes were flooded with the *M. bovis* ELISA positive sera (1/50 dilution) and incubated at 37°C for 2 hours. After the completion of reaction, the membranes were

washed 3-4 times with PBS and 0.1% (v/v) Tween-20, and finally only with PBS. Conjugate (2 ml) horseradish peroxidase 1/1000 dilution (chicken egg albumin 0.1% (w/v) and skim milk 0.1% (w/v) was dissolved in 0.1M PBS, prepared fresh each time) were added to the membranes and were incubated at 37°C for 1 hour. The membranes were again washed 3-4 times with PBS and 0.1% (v/v) Tween-20 and once with only PBS. Substrate nitro blue tetrazolium (2 ml) with alkaline phosphatase conjugate was added to the membranes. The membranes were incubated at room temperature and the development of the band was observed and photographed.

2.20 Substrates, enzymes and chemicals used in the study.

Different substrates like sugars, organic acids, alcohols, enzymes and co-enzymes were used in the study (Table 2.3). Substrates were prepared as a stock solution by dissolving in sterile distilled water and were filter-sterilised (Gelman, 0.2 μm pore size). Substrates were stored as aliquots at –20°C. Enzymes and coenzymes were prepared fresh at the time of use and were kept in ice.

Novel chromogenic substrates (PPR Diagnostics, UK), SLPA-acetate, SLPA-propionate, SLPA-butyrate, SLPA-hexanoate, SLPA-octanoate, and SLPA-decanoate were dissolved in organic solvents (dimethyl sulphoxide, methanol and methoxyethanol). SLPA-phenol (PPR Diagnostics, UK) and 4-methylumbelliferone were used for standard curves. All chemical used were of analytical grade.

All the experiments conducted during the course of project were repeated and the data shown are mean values of up to five replicate experiments.

Table 2.3 Metabolic substrates, enzymes, coenzymes and chemicals used in the study

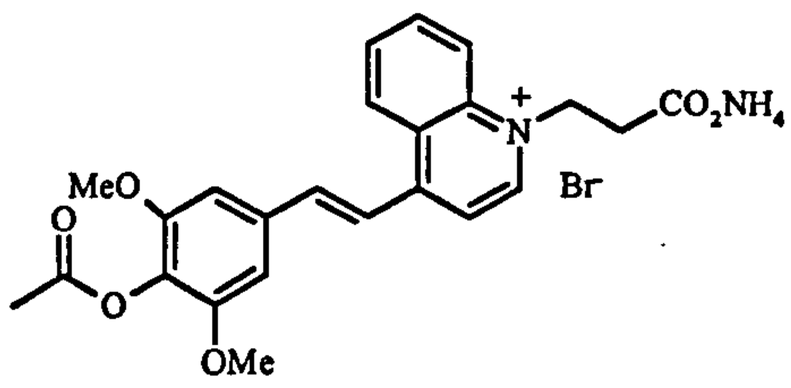
Substrate	Supplier	Code	Substrate	Supplier	Code	Substrate	Supplier	Code
Acetaldehyde	BDH*	27003	Ethyl alcohol	Aldrich	E-702-3	Nicotinamide adenine dinucleotide reduced form (NADH)	Sigma	N-6005
N-acetyl-D-glucosamine	Sigma ●	A-8625	Fructose	Sigma	F-0127	Neutralised liver digest	Oxoid	L-27
Adenosine –5-triphosphate	Sigma	A-7699	Fumarate	Sigma	F-1506	NaH_2PO_4	BDH	10245
Ammonium chloride	BDH	100171	Folin-Ciocalteu	Sigma	F-9252	2-oxobutyrate	Sigma	D-0875
Ammonium persulphate	Sigma	A-3678	L-α-glycerophosphate	Sigma	G-7886	Pyruvate (sodium salt)	Sigma	P-2256
Agarose	Boehringer ♦	1388991	D (+) glucosamine	Sigma	G-4875	Propanol	Sigma	P-6334
Acrylagel	Milford ♠	M-810	Glycerol	Sigma	G-6279	Proteose peptone	Oxoid	L-85
Bisacrylagel	Milford	M-820	Glacial acetic acid	Sigma	A-6283	Potassium chloride	Sigma	P-4505
Bromophenol blue	Sigma	B-8026	Glycine	Milford	EC-405	Ponceau S	Sigma	P-3504
Boric acid	Sigma	B-7660	Glutamine	Sigma	G-3126	Proteinase K	Gibco	25530
Buffer phenol saturated	Gibco ♣	15513-039	Glucose	BDH	G-6936	Phenol-chloroform-isoamyl alcohol	Gibco	N-15593
Bovine or porcine serum	Gibco	26250	Glycerol phosphate oxidase	Sigma	G-4388	Sodium hydroxide	Sigma	S-8045
Blood agar base	Oxoid ♀	CM-271			G0450	Sodium acetate buffer	Sigma	S-2404

Table 2.3 continued

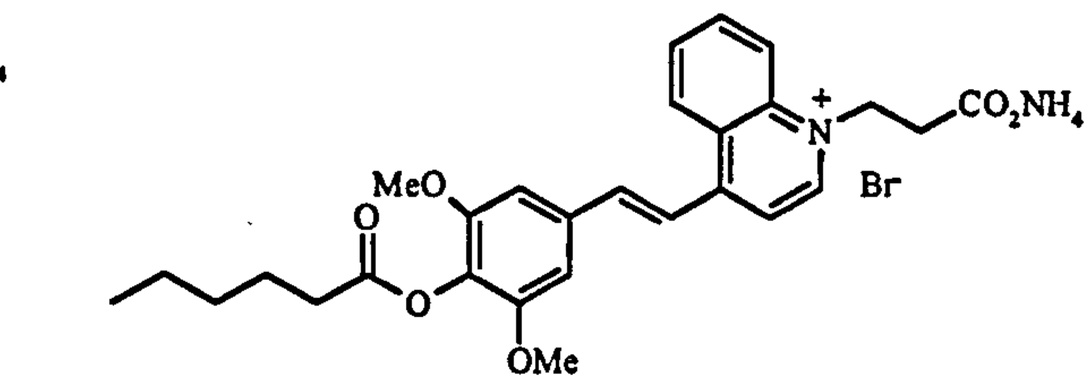
substrate	Supplier	Code	Substrate	Supplier	Code	Substrate	Supplier	Code
Coomassie blue R250	Milford	HS-604	HEPES	Sigma	H-3375	Sodium dodecyl sulphate	Sigma	L-5750
Copper sulphate	Sigma	C-7631	Isopropanol	Sigma	I-0398	Sodium tartrate	Sigma	S-8640
Catalase	Sigma	C 10	Ketoglutarate	Sigma	D-1875	RNase DNase free	Boehrin-ger	1119915
CMRL 1066	Gibco	11530	L-lactate (sodium salt)	Sigma	L-7022	Sodium carbonate	Sigma	S-7795
Diazobicyclo (2.2.2) octane	Aldrich	D-2, 780-2	Lipase(*Candida rugosa*)	Sigma	L-1754	Special peptone	Oxoid	L-72
Dimethyl sulphoxide	Sigma	D-5879	Lactose	BDH	10139	Sodium chloride	Sigma	S-9888
DNA	Sigma	from herring sperm	Malate	Sigma	M-5757	Trehalose	Sigma	T-5251
Disodium hydrogen phosphate	BDH	102494C	Methanol	Sigma	M-3641	Tris base	BDH	443864E
Deoxycholic acid	Sigma	D-6750	Magnesium sulphate	Sigma	M-7929	TEMED	Sigma	T-9281
Ethanol	BDH	10107	Methoxyethanol	Sigma	36,050-3	Tributyrin	Sigma	T-8626
EDTA	Sigma	E-5134	Mycoplasma broth base	Oxoid	CM- 403	Tween-20	Sigma	P-1379
Ethidium bromide	Sigma	E-1510	4-Methyl-umbelliferone	Sigma	M-1381	Vegetable peptones	Oxoid	VG-0100-0300

* BDH, Leicestershire, UK. ● Sigma, Poole, Dorset, UK. ♣ Gibco, Life Technologies, UK. ♠ Milford, national Dignostics, Hull, UK .♀ Oxoid, Basingstoke, Hampshire, UK. ♦ Boehringer, Mannheim, Germany.

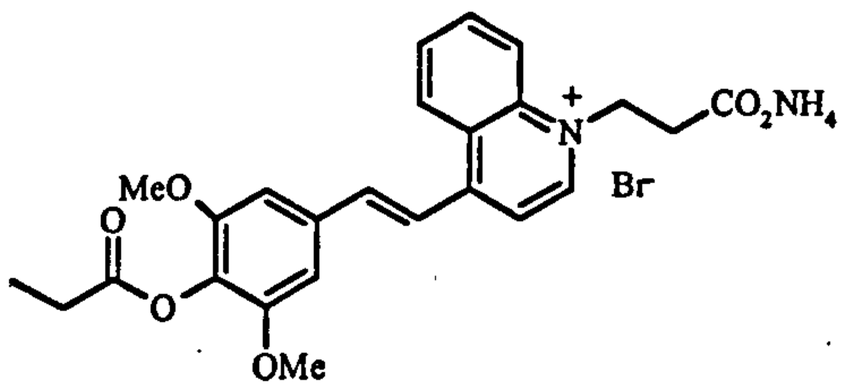
Figure 2.2: Structure of the novel chromogenic substrates used in the study



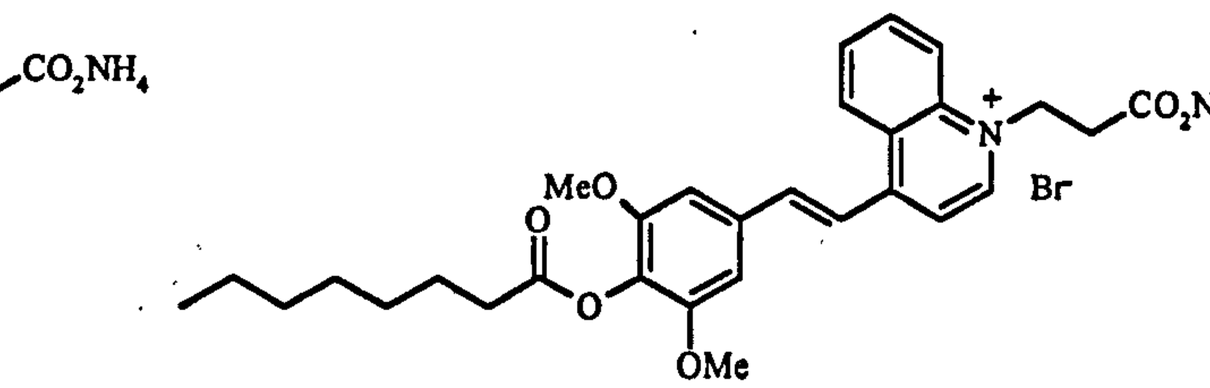
SLPA-acetate



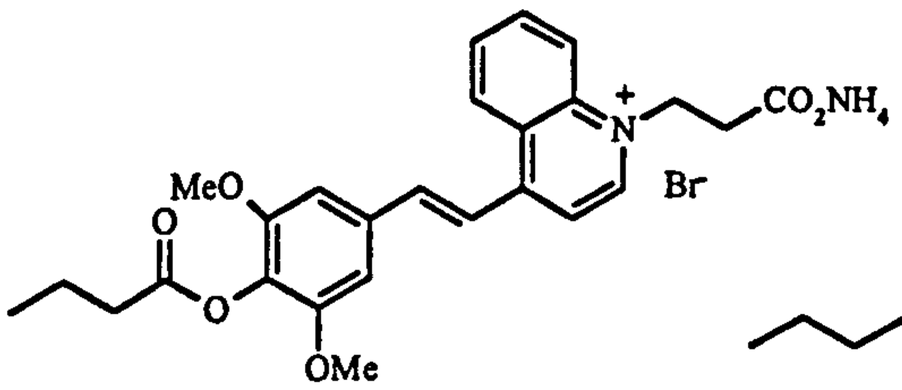
SLPA-hexanoate



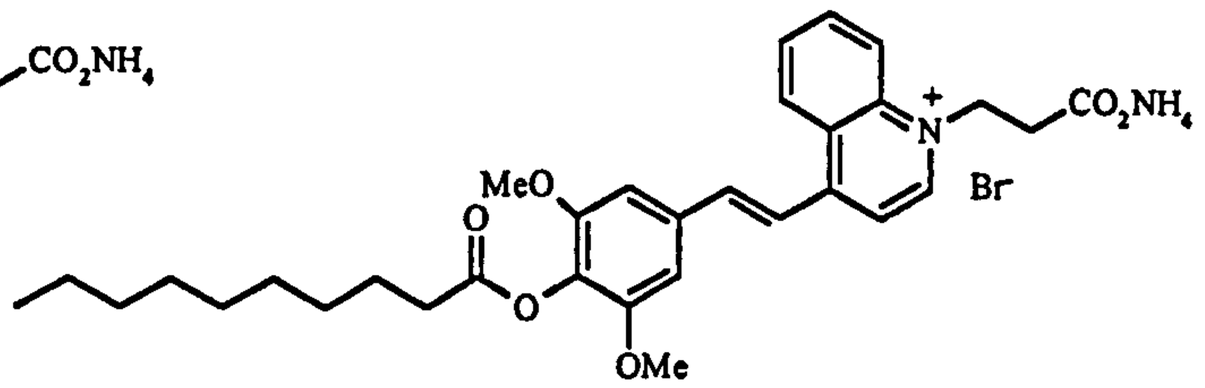
SLPA-propionate



SLPA-octanoate



SLPA-butanoate



SLPA-decanoate

Chapter 3

3. Kinetics of substrate oxidation by *M. agalactiae, M. bovis, M. bovigenitalium* and *M.* ovine serogroup 11

3.1 Introduction

M. agalactiae and *M. bovis* are unable to ferment glucose or hydrolyse arginine and their energy sources for growth appear to be organic acids, for example lactate and pyruvate, which are oxidised to acetate plus carbon dioxide (Miles *et al.*, 1988). *M.* ovine serogroup 11 and *M. bovigenitalium* are also non-fermentative and non-arginine hydrolysing mycoplasmas. *M. agalactiae* and *M. bovis* are closely related and were originally classified within the same species. *M. agalactiae* is the principal cause of contagious agalactia (CA) in sheep and goats in Southern Europe and Asia. *M. bovis* is associated with mastitis, arthritis, and pneumonia, genital and ocular disorders in cattle. Carmichael *et al.* (1972) isolated a mycoplasma from vaginal and preputial samples from Australian sheep presenting clinical pneumonia, characterised and named biotype 2D based on its distinct colony morphology and biochemical characteristics. *M.* ovine serogroup 11, a cause of infertility in sheep, was isolated in the UK for the first time (Nicholas *et al.*, 1999). *M. bovigenitalium* first isolated in the UK from mastitic cattle (Davidson and Stuart, 1960), is biochemically very similar to *M.* ovine serogroup 11 as neither ferment glucose, hydrolyse arginine or possess phosphatase activity; both digest inspissated serum, reduce tetrazolium and produce film and spots, biochemical characteristics which are also shared with *M. agalactiae* and *M. bovis* (DaMassa *et al.*, 1992). Clinically, *M.* ovine serogroup 11 and *M. bovigenitalium* also cause similar reproductive disorders, infertility and impaired sperm motility in their respective hosts (Runhke, 1994).

Ethanol (4mM) caused four-fold stimulation in the rate of oxygen uptake by *Acholeplasma laidlawii* cells suspended in a buffered salts solution (Tarshis *et al.*, 1976). This suggests the presence of alcohol dehydrogenase activity, which would result in the formation of acetaldehyde, a reactive and toxic intermediate. The additional presence of acetaldehyde dehydrogenase would enable oxidation of acetaldehyde to acetate. Bacterial alcohol and

aldehyde dehydrogenases are generally NAD(P)$^+$-dependent (Dixon and Webb, 1958). There is no evidence to indicate that NAD(P)H oxidation in mollicutes is linked to ATP synthesis (Pollack *et al.*, 1997). Himmelreich *et al.* (1996) have revealed by sequence analysis a putative NADP-dependent alcohol dehydrogenase gene in the fully sequenced *M. pneumoniae* genome. There are no published reports of acetaldehyde dehydrogenase activity, or of genome sequence for this function in mollicutes (Pollack *et al.*, 1997). The importance of biochemical data in the assignment of ORF function in genomic sequence and have implications for the phylogenetic distribution of enzymes super families within the prokaryotic kingdom.

The pattern and kinetics of substrate metabolism have only been tested for the type strains of the *M. agalactiae*, *M. bovis*, *M. bovigenitalium* and *M.* ovine serogroup 11, with a limited number of substrates. Importantly, these studies did not include field isolates of these four species. Potential substrates included sugars, organic acids and alcohols whose metabolism would be potentially useful for strain identification. The major aims of the study were to determine the pattern and kinetics of substrate utilisation by field isolates and type strains of the four species of non-fermentative and non-arginine hydrolysing *Mycoplasmas*. In addition, the presence of alcohol dehydrogenase varies amongst *Mycoplasma* species so it may beauseful character in identification. Such information is of potential value in epidemiological studies and in understanding pathogenicity; it might also be applied to the development of improved culture media and rapid biochemical tests aiding identification. Change of cell suspension, DOT or pH provide kinetic data allowing assessment of the likely significance of substrate metabolism at the concentration found in the natural habitat and have been used to identify energy sources which increase growth yield (Miles *et al.*, 1988; Taylor *et al.*, 1994). The methods are also applicable to mollicutes characterisation. Their use has shown major physiological subdivisions between both glucose fermenting and non-fermenting *Mycoplasma* species (Taylor *et al.*, 1994). The pattern of substrate oxidation distinguished specific and sub-specific groups within the *M. mycoides* cluster (Abu–Groun *et al.*, 1994).

The techniques also provide kinetic data allowing assessment of the likely significance of substrate metabolism at the concentration found *in vivo*. Differences in substrate oxidation

patterns between isolates may be related to host or tissue specificity and thus aid understanding of pathogenicity. Biochemical characteristics are used in mycoplasma identification to reduce the number of potential species to which new isolates belong. Identity is then determined using serological or PCR tests. The determination of energy substrates utilised by different species (in addition to arginine and glucose) may enable the development of such tests. In addition, since mycoplasma cell yields for energy substrates are low (Miles, 1992a), large quantities will be consumed during growth. This may have significant effects upon animal hosts as a consequence of both nutrient depletion and the production of toxins (Tryon and Baseman, 1992). Relatively little has been reported on the biochemistry of *M. agalactiae, M. bovis, M. bovigenitalium* and *M.* ovine serogroup 11. In the work reported in this chapter, an extensive study was carried out using newly isolated field strains of four important species, *M. agalactiae, M. bovis, M. bovigenitalium* and *M.* ovine serogroup 11. The reproducibility of the methods was established and a number of specific investigations were carried out to determine the pattern and kinetics of substrate oxidation in:

M. agalactiae (22 European strains)

M. bovis (16 recent isolates from UK)

M. ovine serogroup (12 recent isolates from UK)

M. bovigenitalium (4 strains, 3 from Germany and 1 from UK)

In addition *M. mycoides* SC isolated from experimentally infected animals (in Portugal) were also included in the study.

3.2 Results and discussion

3.2.1 Optimisation and determination of substrate oxidation

The ability of the type and field strains of four species to oxidise a number of potential substrates was determined. Potential substrates such as sugars and organic acids used were as previously described by Miles *et al.* (1988) and Abu-Groun *et al.* (1994). In addition alcohols were also used. The method used for the detection of substrate utilisation was based on measurement of oxygen uptake, detected by changes in DOT.

Initially experiments were conducted and conditions were optimised. Exponential phase cultures were used for experiments. The time for harvesting of cultures used was between 18-24 hours depending upon the *Mycoplasma* strain. The optimum harvesting time for cell suspensions was determined by using different ages of cultures for substrate oxidation. Type and field strains of *M. agalactiae* and *M. bovis* were used in the study. Pyruvate and isopropanol oxidation was monitored every 24 hours. The initial rates of oxygen uptake by *M. agalactiae* type strain and strain 453/94 after 24 hours were 52-73 for pyruvate and 149-258 nmol/min/mg cell protein for isopropanol. After 72 hours the rates of oxidation for pyruvate and isopropanol were 5-8 and 7-19 nmol/min/mg cell protein respectively. Similar results were obtained for *M. bovis* type and field strain 79B96 whose rates of oxidation for pyruvate and isopropanol when harvested at 24 hour were 47-57 and 188-209 nmol/min/mg cell protein respectively. The rates of oxidation after 72 hours were very low (pyruvate, 7-11 nmol/min/mg cell protein and isopropanol, 9 nmol/min/mg cell protein). The metabolic activity of the *M. agalactiae* and *M. bovis* cell suspension was maximum in exponential phase and it was significantly decreased after 72 hours (Table 3.1). The time of washing of cells and resuspension was kept to less than 30 mins and cell suspensions were used immediately.

Cell suspensions were washed twice in Ringer-HEPES buffer. Optimum buffer pH was determined by washing and resuspending cell suspensions in different Ringer-HEPES buffers in the pH range from 6.8-8. Pyruvate oxidation was monitored. It was determined that the optimum pH for the cell suspensions washing and resuspension was 7.6. Pyruvate oxidation was 54 nmol/min/mg cell protein in Ringer-HEPES pH 7.6 (Table 3.2). The OD of the cell suspension was adjusted to 1 which was approximately equivalent to 250 μg cell protein ml^{-1} and a viable count of 10^9 cfu ml^{-1}. When substrates were added to the cell suspensions, there was instantaneous reduction in DOT. In the absence of added substrate, oxygen uptake was not detected. The rate of oxygen uptake did not decline until the substrate was completely utilised. In this way the metabolism of a number of substrates was detected, using a single cell suspension. When the DOT of the cell suspension was reduced to a low level, the system was reoxygenated. The substrates used were: L-lactate, pyruvate, 2-oxobutyrate (100 μM), sugars (25 μM-2.5mM) and alcohols (100 μM-10 mM). The rates of substrate metabolism for *M. bovis, M. agalactiae, M. bovigenitalium* and, *M.* ovine

serogroup 11 were expressed in relation to the rate for pyruvate metabolism but in the case of *M. mycoides* SC this was expressed relative to glucose. The substrate used at the start of experiment was used again at a later stage to confirm that the activity of cells had not declined.

3.2.2 Determination of affinity constants K_s and V_{max} values

The values of saturation constants (K_s) and maximum velocity (V_{max}) of substrate utilisation were determined from chart recorder tracings of change in DOT with time, following the addition of substrate. The maximum rates of substrate utilisation (V_{max}) were determined from initial rates of oxygen uptake and in calculating K_s and $V_{max;}$ it was assumed that Michaelis kinetics was observed.

$$V = \frac{V_{max}.S}{K_s + S}$$

Where 'V' is the rate of substrate utilisation (the rate of O_2 uptake or the rate of pH change) and 'S' is the substrate concentration. Generally the straight line of best fit was drawn in plots of 1/V against 1/S and K_s was the reciprocal of the intercept on the x-axis and V_{max} was the reciprocal of the intercept on y-axis. It is assumed that the DOT or change in pH at any time 't' after the addition of the substrate was proportional to the substrate used and the substrate consumed was proportional to oxygen taken up; therefore the substrate 's' at time 't' was,

Initial substrate concentration x (a-b)/a

Where 'a' was the total reduction in DOT or pH change following the addition of substrate and 'b' the change in DOT at time 't' (Figure 3.1). The values for the affinity constant K_s and maximum velocity V_{max} could be estimated from recorder tracings, if the affinity of the substrate was high (Figure 3.1). This method is based on the assumption that reduction in DOT (r) is initially constant and the metabolism at maximum rate is equal to:

$$V_{max} = r.\frac{\text{Initial substrate concentration}}{a}$$

The point where the rate of metabolism has declined to r/2 may be estimated and which is proportional to x/2. The K_s values are half of the V_{max} values and is equal to:

$$K_s = \frac{\text{x. Initial substrate concentration}}{\text{a}}$$

In cases where the affinity for the substrate was low, the rates of oxygen uptake were determined at different substrate concentrations. Affinity constant values were determined from the double reciprocal plots of the rate of substrate utilisation against substrate concentrations (Miles and Agbanyim, 1998).

3.2.3 Patterns and kinetics of substrate oxidation by *M. agalactiae*, *M. bovis*, *M. bovigenitalium* and *M.* ovine serogroup 11

Substrate oxidation was investigated in 54 strains of four important species of non-fermentative and non-arginine hydrolysing mycoplasmas which were newly isolated from the UK and Europe. Patterns and kinetics of substrate oxidation are summarised in Tables 3.3-3.8. Substrate oxidation was determined from changes in DOT as described in Chapter 2. The relative rates of oxygen uptake are presented as a % of that for pyruvate.

3.2.3.1 Oxidation of organic acids

All *M. agalactiae* strains oxidised organic acids at relatively high rates. The relative rates (% of pyruvate) of oxidation were 30-262 % for L-lactate and 45-166% for 2-oxobutyrate (Table 3.3). The K_s values were in the range of 5-12 μM for L-lactate, 7-23 μM for pyruvate and 6-26 μM for 2-oxobutyrate (Table 3.4). Organic acids were oxidised by all the *M. agalactiae* strains at high rates and with low K_s values showing high affinity for the substrates (Figure 3.4).

All strains of *M. bovis* tested for organic acid oxidation showed similar patterns to that of *M. agalactiae*. The relative rates (% of pyruvate) of oxidation for L-lactate and 2-oxobutyrate were 22-112 % and 57-130 % respectively (Table 3.5). The K_s values for the organic acids were also low: L-lactate 5-14 μM, pyruvate 8-13μM and for 2-oxobutyrate,

4-13 μM (Table 3.6) showing the highest affinity for these substrates. All sixteen strains of *M. bovis* showed highest relative rates (% of pyruvate) of the oxygen uptake for organic acids (Figure 3.5).

All sixteen *M. bovigenitalium* and *M.* ovine serogroup 11 strains also oxidised organic acids with high rates and low K_s values showing high affinity for the substrates. Organic acids oxidised were with relative rates (% of pyruvate), L-lactate, 67-221 % and 2-oxobutyrate, 74-132 % (Table 3.7). All strains showed high affinity for organic acids. The K_s values were in the range of 5-13 μM for L-lactate, 6-12 μM for 2-oxobutyrate and 7-14 μM for pyruvate (Table 3.8). Rates of oxygen uptake with L-lactate, 2-oxobutyrate and pyruvate were high with low K_s value indicating high affinity (Figures 3.6 and 3.7).

All strains of *M. agalactiae, M. bovis, M. bovigenitalium*, and *M.* ovine serogroup 11 oxidised organic acids (100 μM) at high rates, with low K_s values and subsequently high affinity. These results are in agreement with Wadher *et al.* (1990) who found that some non-fermentative non-arginine-hydrolysing mycoplasmas like *Mycoplasma agalactiae, Mycoplasma bovis* and *Mycoplasma californicum* oxidised organic acids. Organic acid oxidation was also found in *Mycoplasma gallinarum, Mycoplasma columbinsale* and *Mycoplasma columbinum* (Taylor *et al.*, 1994). Miles *et al.* (1994) found *M. bovis* was unable to ferment glucose or hydrolyse arginine but obtained energy from oxidation of organic acids. It was observed in this study that lactate oxidation appeared saturated at low concentrations (50 μM). The K_s values for L-lactate were low for all the strains indicating high affinity.

Lee *et al.* (1986) reported evidence for a specific lactate/pyruvate transport protein in *Mycoplasma mycoides*, which would presumably be saturated by relatively low substrate concentrations. Abu-Groun *et al.* (1994) also reported that lactate oxidation was generally detected at low concentrations. It is probable that at high lactate concentrations, uptake of undissociated lactic acid may occur by passive diffusion. Miles *et al.* (1985) have reported K_s values of 20 μM for lactate oxidation. Lactate production and /or metabolism is a feature of many mollicutes and lactate dehydrogenase (LDH) has been reported to be

present in all species, including non-glycolytic species (Allsop and Mathews, 1975; Pollack *et al.*, 1989) with the exception of ureaplasmas (Cocks *et al.*, 1985).

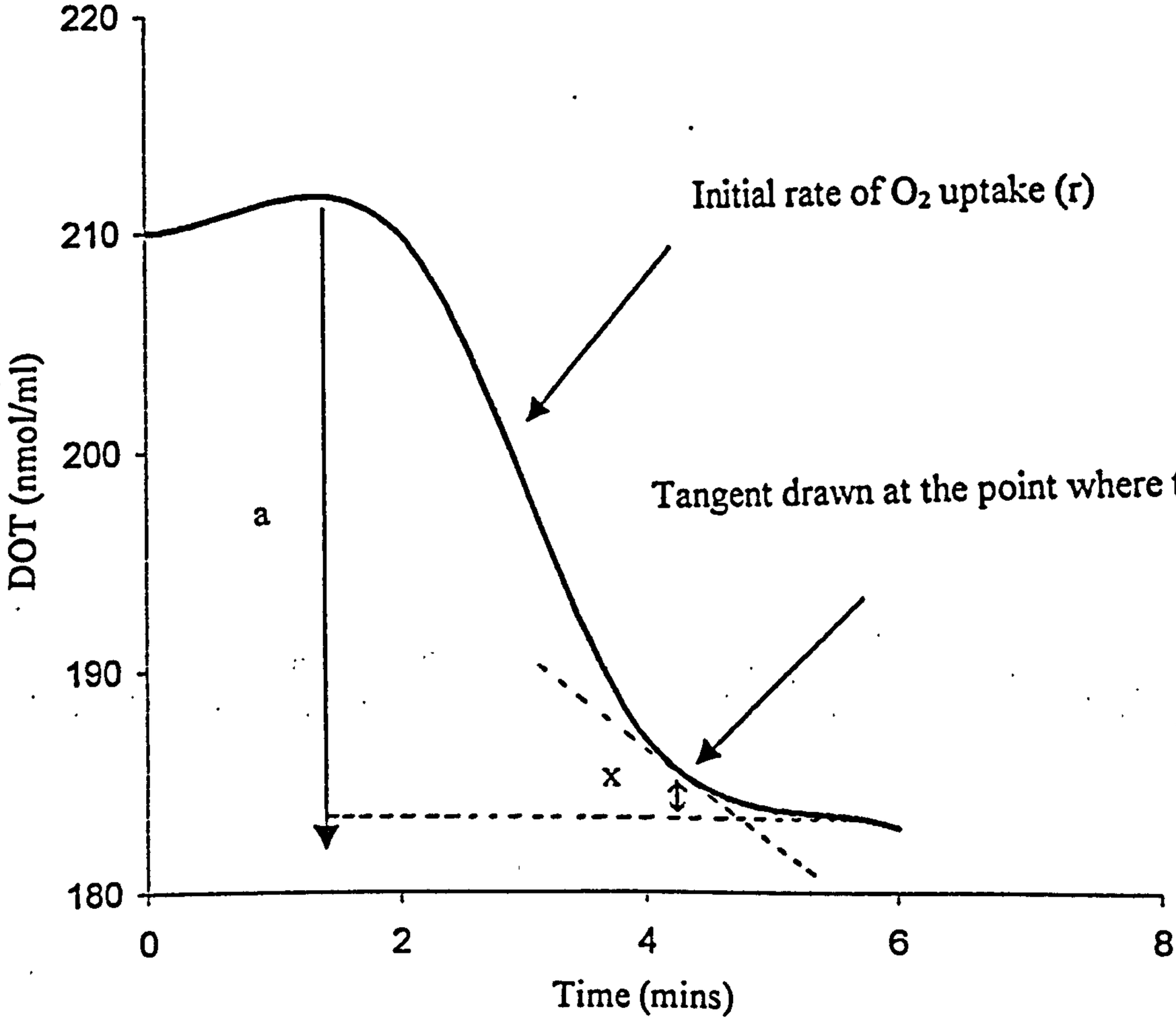
3.2.3.2 Oxidation of alcohols

The ability of ethanol to stimulate oxygen uptake has been reported in *Acholeplasma* and in some *Mycoplasma* species (Abu-Groun, 1992). Ethanol dehydrogenase (EDH) activity has also been reported in *Acholeplasma* and *Mycoplasma* species by Salih *et al.* (1983). It is not clear why *Acholeplasma* and *Mycoplasma* should possess alcohol dehydrogenase (ADH) activity since ethanol oxidation would not be predicted to lead to ATP synthesis. If the presence of alcohol dehydrogenase varies amongst *Mycoplasma* species, it might be a useful character in identification, therefore an investigation of metabolism of alcohols by a range of newly isolated strains of *M. agalactiae*, *M. bovis*, *M.* ovine sergroup 11 and *M. bovigenitalium* was carried out.

All *M. agalactiae* strains oxidised isopropanol at very high rates and this substrate was oxidised at relatively high rates when added at only 25 μM concentration. The relative rates (% of pyruvate) were 65-937 % for isopropanol, while propanol was oxidised at very low rates 5-30%, and in strains 2193/91, 1070/93, 1209/93, 471/93, 499/93, 314/97, 423/98, 4400/99, 2245/99 and LF/00 propanol oxidation was not detected even at higher concentrations (10 mM). Ethanol and acetaldehyde (100 μM) were also metabolised by *M. agalactiae* strains at low rates 6-36 % and 6-33 % respectively. Strains 423/98, 4400/99 and 2245/99 did not oxidise ethanol at higher concentrations (1mM). Acetaldehyde was also not detected in strain 314/97 and 423/98 (Table 3.3). All strains of *M. agalactiae* showed low K_s values for isopropanol, 15-27 μM, high K_s values for propanol, 155 μM-10 mM, ethanol, 103 μM-1 mM and for acetaldehyde; the values of K_s were 270 μM-1 mM (Table 3.4).

Alcohol oxidation by *M. bovis* was also high. Isopropanol was oxidised at very high rates compared to other alcohols. The relative rates of oxygen uptake for isopropanol, was 174-953 %.

Figure 3.1 Calculation of K_s and V_{max} values for substrate utilisation from dissolved oxygen tension (DOT) curve versus time. The representative curve shows the complete oxidation of pyruvate (50 μM) by *Mycoplasma bovis* strain 193B96.



$$K_s = \frac{x \,.\, \text{Initial substrate concentration}}{a}$$

$$V_{max} = \frac{r \,.\, \text{Initial substrate concentration}}{a}$$

Table 3.1 The effect of culture age on the metabolic activity of mycoplasma cell suspensions in Ringer-HEPES buffer. Substrate oxidation by *Mycoplasma agalactiae* type strain NCTC 10123, 453/94 and *Mycoplasma bovis* type strain NCTC 10131, 79B96. Initial rates of oxygen uptake (nmol/min/mg cell protein).

Strain code	**Time (hr)**	**Optical density (540 nm)**	**Pyruvate (nmol/min/mg cell protein)**	**Isopropanol (nmol/min/mg cell protein)**
Mycoplasma agalactiae NCTC 10123	24	0.232	52	149
	48	0.194	18	16
	72	0.187	5	7
453/94	24	0.255	73	258
	48	0.203	17	48
	72	0.194	8	19
Mycoplasma bovis 10131	24	0.222	57	188
	48	0.208	30	32
	72	0.174	11	9
79B96	24	0.264	47	209
	48	0.231	18	25
	72	0.200	7	9

Table 3.2 The effect of Ringer-HEPES buffer pH on metabolic activity of *M. bovis* type strain NCTC 10131. Pyruvate oxidation was monitored by DOT.

Strain code	**Initial rates of oxygen uptake (nmol/min/mg cell protein)**			
M. bovis NCTC 10131	pH 6.8	pH 7.0	pH 7.6	pH 8.0
	27	33	54	32

Propanol was oxidised at low rates 8-38 %. The relative rates of oxygen uptake for ethanol and acetaldehyde were also low, 6-26 % and 13-70 % respectively. *M. bovis* NCTC 10131 type strain did not oxidise ethanol at high concentration (1mM) (Table 3.5).

Low K_s values for isopropanol, 12-27 μM were shown by all the *M. bovis* strains. The K_s values for propanol were 139 μM-10 mM, for ethanol, 103 μM-1 mM and for acetaldehyde were 143 μM-1 mM. All these K_s values for ethanol, acetaldehyde and propanol were high compared to isopropanol and showed lower affinity for these substrates (Table 3.6).

M. bovigenitalium and *M.* ovine serogroup 11 also showed similar patterns of alcohol oxidation and the relative rates of isopropanol by all the strains were high 102-1600 %. Propanol, ethanol and acetaldehyde were also oxidised at low rates with relative rates of oxygen uptake (% of pyruvate) being 10-37 %, 13-63 % and 15-45 % respectively. *M. bovigenitalium* strain 57B00 did not oxidise propanol, ethanol or acetaldehyde at higher concentrations. *M.* ovine serogroup 11 strains, 50SR98, 52SR98, 95SR99, 96SR99, 126SR99 and 129SR99 did not oxidise ethanol at high concentrations (1 mM). Acetaldehyde metabolism was not detected by strains 50SR98, 52SR98, 129SR99 and strain 2D at high concentration (Table 3.7).

The values of K_s for isopropanol by *M. bovigenitalium* and *M.* ovine serogroup 11 were also low 16-29 μM compared to other alcohols. The K_s values for propanol by all the strains were 360 μM-8 mM, for ethanol, 30 μM-1 mM. The K_s values for acetaldehyde were 205 μM-1 mM. All strains of the four species have a similar pattern of alcohol oxidation with high affinity towards isopropanol, low affinity towards other alcohols, with the exception of some strains which were unable to metabolise ethanol, propanol and acetaldehyde.

Since K_s values for isopropanol were low, it was possible under the experimental conditions used to monitor its complete oxidation by all these strains. Oxygen uptake following addition of isopropanol (100 μM) ceased quickly because of substrate exhaustion. When further substrate was added oxygen uptake was renewed. The mean total oxygen uptake (five experiments) was 0.5 mol per mol of isopropanol.

Table 3.3 Biochemical characteristics of *M. agalactiae* (European strains). `Relative rates of oxygen uptake (% of pyruvate). Concentration of the substrates used were: L-lactate, 2-oxobutyrate, pyruvate, isopropanol, (all at 100 μM), propanol, ethanol and acetaldehyde (all at 100 μM-10 mM)

Strain code	Substrate						
	L-lactate	**2-oxobutyrate**	**Pyruvate**	**Isopropanol**	**Propanol**	**Ethanol**	**Acet-aldehyde**
NCTC 10123	133	111	100	282	8	20	27
2193/91	150	166	100	399	Nd	21	Nt
723/93	30	45	100	368	30	11	Nt
499/93	132	141	100	82	Nd	7	7
1070/93	175	144	100	138	Nd	10	Nt
1209/93	104	129	100	158	Nd	33	Nt
471/93	86	73	100	116	Nd	18	Nt
453/94	62	96	100	327	6	18	Nt
101/94	72	95	100	289	7	6	Nt
730/97	82	118	100	129	12	24	Nt
314/97	64	104	100	133	Nd	15	Nd
423/98	72	77	100	65	Nd	Nd	Nd
4400/99	165	96	100	204	Nd	Nd	Nt
2245/99	60	95	100	87	Nd	Nd	Nt
1536/99	89	102	100	276	13	15	9
LF/00	116	113	100	135	Nd	19	9
4 a	255	100	100	772	14	14	Nt
10 a	262	104	100	937	8	25	33
11 b	205	95	100	352	6	32	6
6 gb	175	106	100	255	9	17	Nt
A. Vet	147	98	100	162	5	15	Nt
33328	149	91	100	230	14	36	7

Nd, metabolism not detected. Nt, not tested.

Table 3.4 Biochemical characteristics of *M. agalactiae* (European strains). K_s values (μM).

Strain code	Substrate						
	L-lactate	**2-oxobutyrate**	**Pyruvate**	**Isopropanol**	**Propanol**	**Ethanol**	**Acet-aldehyde**
NCTC 10123	8	10	9	15	8 mM	103	270
2193/91	9	14	15	18	Nd	900	Nt
723/93	9	26	23	19	1.3 mM	1 mM	Nt
499/93	9	12	13	15	Nd	1 mM	1 mM
1070/93	9	11	13	18	Nd	1 mM	Nt
1209/93	10	10	12	20	Nd	629	Nt
471/93	9	10	13	19	Nd	129	Nt
453/94	12	13	12	25	2 mM	1 mM	Nt
101/94	11	14	14	21	4.5 mM	1 mM	Nt
730/97	10	9	12	23	4.6 mM	355	Nt
314/97	8	10	13	16	Nd	129	Nt
423/98	12	11	11	19	Nd	Nd	Nd
4400/99	7	11	11	14	Nd	Nd	Nd
2245/99	10	9	11	23	Nd	Nd	Nt
1536/99	9	10	8	16	4.1 mM	1 mM	1 mM
LF/00	10	12	14	27	Nd	1 mM	1 mM
4 a	8	7	8	14	3.9 mM	350	1 mM
10 a	8	9	9	15	4.6 mM	226	1 mM
11 b	7	6	8	15	5.6 mM	201	1 mM
6 gb	7	7	7	8	6.2 mM	1 mM	Nt
A. Vet	5	7	8	15	10 mM	331	Nt
33328	7	8	9	14	155	157	810

Nd, metabolism not detected. Nt, not tested.

Table 3.5 Biochemical characteristics of *M. bovis*. Relative rates of oxygen uptake (% of pyruvate). Concentrations of the substrate used were: L-lactate, 2-oxobutyrate, pyruvate, isopropanol, (all at 100 μM), propanol, ethanol and acetaldehyde (all at 100 μM-10 mM)

Strain code	Substrate						
	L-lactate	2-oxobutyrate	Pyruvate	Isopropanol	Propanol	Ethanol	Aceta-ldehyde
NCTC 10121	112	89	100	376	12	Nd	16
79B96	86	113	100	462	37	9	Nt
81B96	86	102	100	370	19	7	36
82B86	79	96	100	282	31	6	24
119B96	60	57	100	423	14	25	21
193B96	22	74	100	174	38	9	70
67M98	88	83	100	366	15	13	28
135B99	85	127	100	596	19	19	18
136B99	89	124	100	953	13	13	26
137B99	86	95	100	590	11	22	Nt
139B99	84	123	100	587	19	17	25
142B99	76	87	100	694	18	17	Nt
156B99	87	82	100	798	9	9	Nt
5B00	112	116	100	712	12	26	13
10B00	29	92	100	261	8	21	Nt
12B00	68	130	100	475	31	56	Nt

Nd, metabolism not detected. Nt, not tested.

Table 3.6 Biochemical characteristics of *M. bovis*. K_s values (μM).

Strain code	Substrate						
	L-lactate	**2-oxobutyrate**	**Pyruvate**	**Isopropanol**	**Propanol**	**Ethanol**	**Acet-aldehyde**
NCTC 10121	12	10	8	27	1.6 mM	Nd	810
79B96	7	9	11	20	4.3 mM	1 mM	Nt
81B96	11	8	11	21	2.6 mM	683	143
82B86	9	11	13	25	1.9 mM	450	156
119B96	10	4	10	16	2 mM	450	186
193B96	14	11	13	21	1.6 mM	786	326
67M98	11	7	10	19	5.1 mM	1 mM	932
135B99	5	9	10	20	150	586	1 mM
136B99	5	7	11	12	197	900	607
137B99	6	9	10	15	139	855	Nt
139B99	9	8	10	19	6.4 mM	465	528
142B99	11	9	9	20	1.5 mM	303	Nt
156B99	8	8	10	24	360	618	Nt
5B00	11	12	13	18	7.2 mM	185	1 mM
10B00	7	10	13	18	4.7 mM	103	Nt
12B00	9	13	13	20	10 mM	302	Nt

Nd, metabolism not detected.

Nt, not tested.

Table 3.7 Biochemical characteristics of *M. bovigenitalium* and *M.* ovine serogroup 11. Relative rates of oxygen uptake (% of pyruvate). Concentrations of the substrate used were: L-lactate, 2-oxobutyrate, pyruvate, isopropanol, (all at 100 μM), propanol, ethanol and acetaldehyde (all at 100 μM-10mM)

Strain code	Substrate						
	L-lactate	2-oxobutyrate	Pyruvate	Isopropanol	Propanol	Ethanol	Acet-aldehyde
NCTC 10122	80	85	100	1600	30	38	30
398/87	221	128	100	213	36	61	19
434/81	158	74	100	291	13	13	45
57B00	97	132	100	287	Nd	Nd	Nd
Type strain 2D	137	83	100	102	37	36	Nd
48SR99	70	91	100	410	32	62	42
50SR99	67	87	100	118	17	Nd	Nd
52SR98	69	101	100	147	21	Nd	Nd
3SR99	128	91	100	263	33	30	40
47SR99	97	107	100	551	22	53	23
48SR99	116	103	100	201	24	63	15
52SR99	107	103	100	383	18	36	Nt
95SR99	70	121	100	126	10	Nd	Nt
96SR99	156	122	100	327	20	Nd	20
126SR99	109	98	100	313	22	Nd	Nt
129SR99	99	107	100	189	11	Nd	Nd

Nd, metabolism not detected.

Nt, not tested.

Table 3.8 Biochemical characteristics of *M. bovigenitalium* and *M.* ovine serogroup 11. K_s values (μM).

Strain code	Substrate						
	L-lactate	2-oxobutyrate	Pyruvate	Isopropanol	Propanol	Ethanol	Acet-aldehyde
NCTC 10122	13	11	10	21	4.4 mM	50	1 mM
398/87	13	12	12	29	1.1 mM	1 mM	205
434/81	9	8	7	23	2 mM	1 mM	1 mM
57B00	11	11	14	21	Nd	Nd	Nd
Type strain 2D	5	9	10	18	450	450	Nd
48SR99	13	10	14	21	2.5 mM	70	1 mM
50SR99	7	10	8	24	1.3 mM	Nd	Nd
52SR98	12	9	12	25	640	Nd	Nd
3SR99	9	12	11	24	2.6 mM	280	1 mM
47SR99	9	10	8	24	2.3 mM	30	430
48SR99	10	11	11	26	2 mM	1 mM	1 mM
52SR99	6	9	10	16	1.2 mM	400	Nt
95SR99	7	9	8	21	360	Nd	Nt
96SR99	6	11	14	19	1.5 mM	Nd	1 mM
126SR99	10	7	11	18	930	Nd	Nt
129SR99	7	6	8	23	8 mM	Nd	Nd

Nd, metabolism not detected.

Nt, not tested.

The high affinity of isopropanol (low K_s values) and low affinity (high K_s values) for other alcohols such as ethanol and propanol might be important, as substrates with high K_s values may not be present in sufficient concentrations *in vivo* to be utilised at significant rates. Furthermore, where affinity for the substrates is low, *Mycoplasma* strains may not be specifically adapted to their utilisation.

Oxidation of ethanol and organic acids indicated alternate modes of metabolism might be useful in taxonomy (Taylor *et al.*, 1996). The likely end product of ethanol metabolism is acetate, and oxidation presumably occurs via NAD^+ reduction with no energy conservation. The oxidation of isopropanol at a high rate and high affinity by all the strains is a distinctive ability of these species. The significance of alcohol and isopropanol oxidation is unclear, as it would not be predicted to lead to ATP generation. One possibility is that alcohol oxidation is a factor in pathogenicity leading to the accumulation of aldehydes and ketones. Isopropanol oxidation consumed 0.5 mol oxygen per mol which was consistent with metabolism to acetone. The tentative function assigned to the alcohol dehydrogenase gene in *M. pneumoniae* is the reduction of acetaldehyde, formed during the catabolism of 2-deoxyribose-5-phosphate to glyceraldehyde-3-phosphate (Himmelreich *et al.*, 1996). It was also considered possible that the enzyme was important in regulating $NADP^+$/NADPH ratio which may be significant in identification of this enzyme in *M. agalactiae*, *M. bovis*, *M. bovigenitalium* and *M.* ovine serogroup 11.

Alcohol dehydrogenases are fairly non-specific with respect to the alcohol substrate at any particular alcohol concentration (Dixon and Webb, 1958); the highest rates of oxidation were always for isopropanol. Since isopropanol is a secondary alcohol, the product will be ketone (acetone) rather than an aldehyde. Mycoplasmas have restricted metabolic pathways, and the ability of these species to oxidise alcohols at high rates was unexpected. There may be a role for alcohol dehydrogenase in removing trace quantities of acetaldehyde but it will not lead to ATP synthesis. The origin of alcohols oxidised *in vivo* is unclear. Presumably alcohols are available, otherwise the capacity to oxidise them would have been lost. Alcohols are possibly produced by fermentative micro-organisms which make up the normal flora of the gut and are able to diffuse throughout the host tissues. It is possible that alcohol oxidation is associated with pathogenicity due to the accumulation of aldehyde. In *M.*

agalactiae, M. bovis, M. bovigenitalium and *M.* ovine serogroup 11 which are all pathogens of ruminants, the high affinity of isopropanol indicates that the principal role of alcohol dehydrogenase may be the oxidation for isopropanol to acetone. For strains of all these species, maximum isopropanol oxidation rates were approximately five-fold greater than lactate and pyruvate (Figures 3.2 and 3.3).

All strains were unable to oxidise glycerol (2.5 mM), showing that they lack appropriate enzymes. Glycerol oxidation is found in members of the *Mycoplasma mycoides* cluster (Houshaymi *et al.*, 1997). These results suggested that the pattern of alcohol oxidation might be of value in mollicutes characterisation and identification.

3.2.3.3 Oxidation of sugars

Cell suspensions of *M. agalactiae, M. bovis, M. bovigenitalium* and *M.* ovine serogroup 11 were tested for the ability to oxidise a wide range of sugars which had been reported to be utilised by the members of *Mycoplasma mycoides* cluster (Abu-Groun *et al.*, 1994) and other *Mycoplasma* groups. The sugars tested were N-acetylglucosamine, fructose, glucosamine, glucose, maltose, mannose, sucrose and trehalose (2.5 mM). None of the test strains metabolised sugars possibly because they lack much of the EMP pathway, enzymes such as hexokinase, phosphofructokinase and aldolases.

It was assumed that non-fermentative strains did not contain enzymes for the phosphorylation of sugars. The inability to oxidise sugars show that mycoplasmas have limited biosynthetic activity. The type strain F38 and two further subspecies of *capripneumoniae* are different from all other strains, as they do not oxidise glucose, so they are presumably lacking both the PEP:PTS system and much of the EMP pathway (Bonnet *et al.*, 1993). PEP:PTS activity in the non-fermentative mycoplasmas is absent (Cirillo, 1979). The absence of phosphofructokinase and aldolase activities in non-fermentative species and their presence in fermentative *Mycoplasma* sp. suggests that these enzymes may be useful in a comparative study of the molecular divergence of enzymes and the phylogeny of these genera (Fothergill-Gilmore, 1986). Miles (1992b) reported that *M. hominis* and *M.*

bovigenitalium lacked hexokinase, phosphofructokinase (PFK) and fructose –1, 6-bisphosphate aldolase.

3.2.3.4 Oxidation of citric acid cycle intermediates.

All strains of the four species were unable to oxidise the citric acid cycle intermediates, fumarate, malate and 2-oxoglutarate (1mM). These strains lack the citric acid cycle and mollicutes are known to be devoid of cytochromes (Pollack, 1979). In those mollicutes studied, there is no convincing evidence for a functional citric acid cycle, though representative members of all groups, other than *Acholeplasma,* possess malate dehydrogenase (Manolukas *et al.*, 1988; Pollack *et al.*, 1989). So it was confirmed that all tested species of non-fermentative and non-arginine hydrolysing mycoplasmas did not contain citric acid cycle enzymes.

3.3 Substrate utilisation by *M. mycoides* subsp. *mycoides* SC strains.

The sugars and organic acids tested as metabolic substrates included all those substrates which were previously shown to be oxidised by members of the *M. mycoides* cluster in the study of Abu-Groun *et al.* (1994). Substrate oxidation was studied in seven *M. mycoides* subsp. *mycoides* SC strains and one large colony (LC) strain. These strains were isolated from experimentally infected animals from Europe and one strain was isolated from Africa. The aim of this study was to classify these strains on the basis of substrate oxidation. All these isolates were from cattle and were cloned by selection of single colonies on blood agar plates.

3.3.1 Oxidation of glucose

All strains used in this study were able to ferment glucose. In glucose-fermenting species, the EMP is present and lactate formed by the reduction of pyruvate is the major product of fermentation under anaerobic conditions. Under aerobic conditions, pyruvate is oxidised to acetate plus CO_2 in a series of reactions, which lead to the synthesis of an additional 1 mol of ATP per mol pyruvate (Miles, 1992b).

Figure 3.2 Rates of oxygen uptake by *M. bovis* and *M. agalactiae* type and field strains.

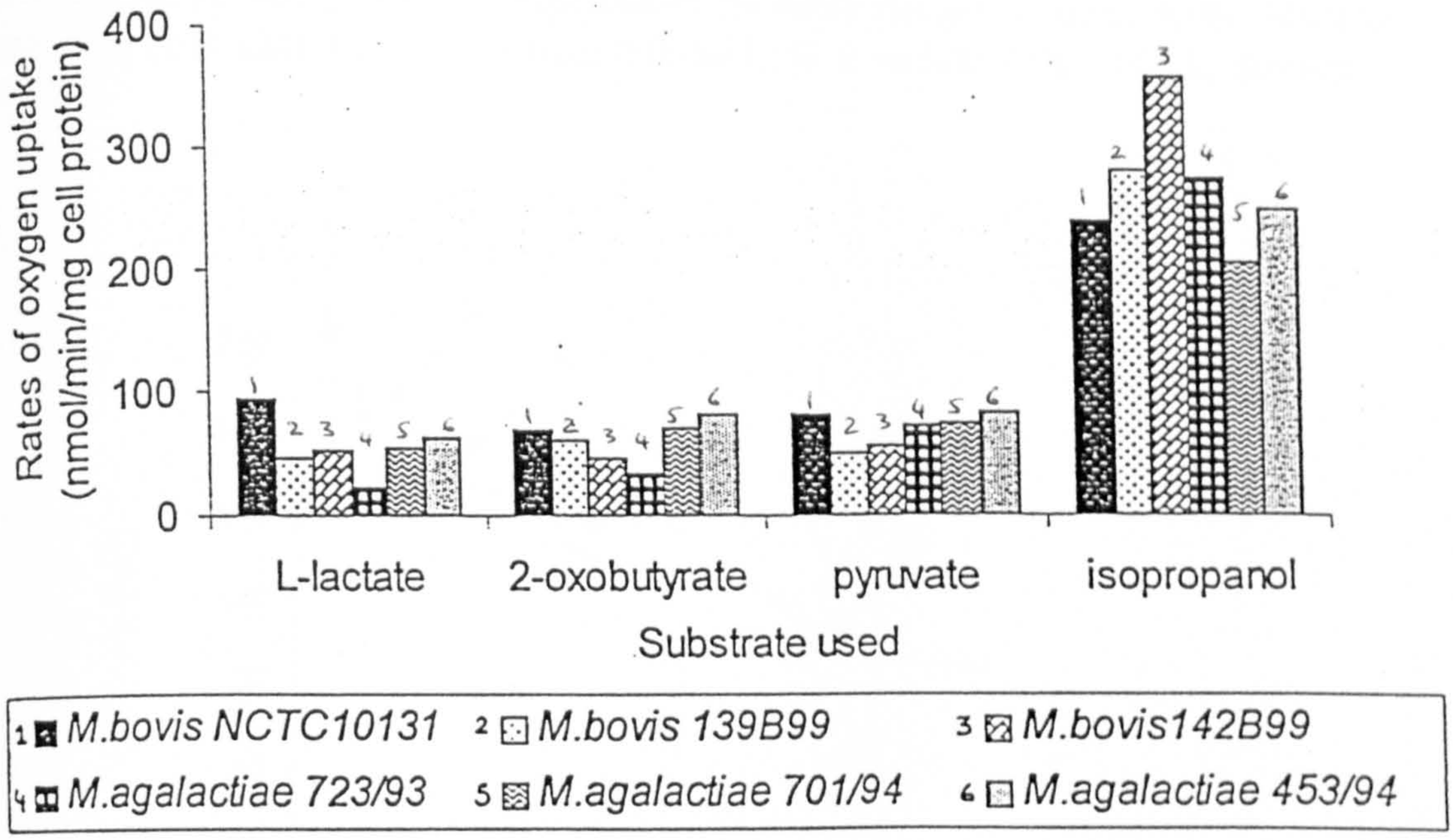


Figure 3.3 Rates of oxygen uptake by *M.* ovine serogroup *11* and *M. bovigenitalium* type and field strains.

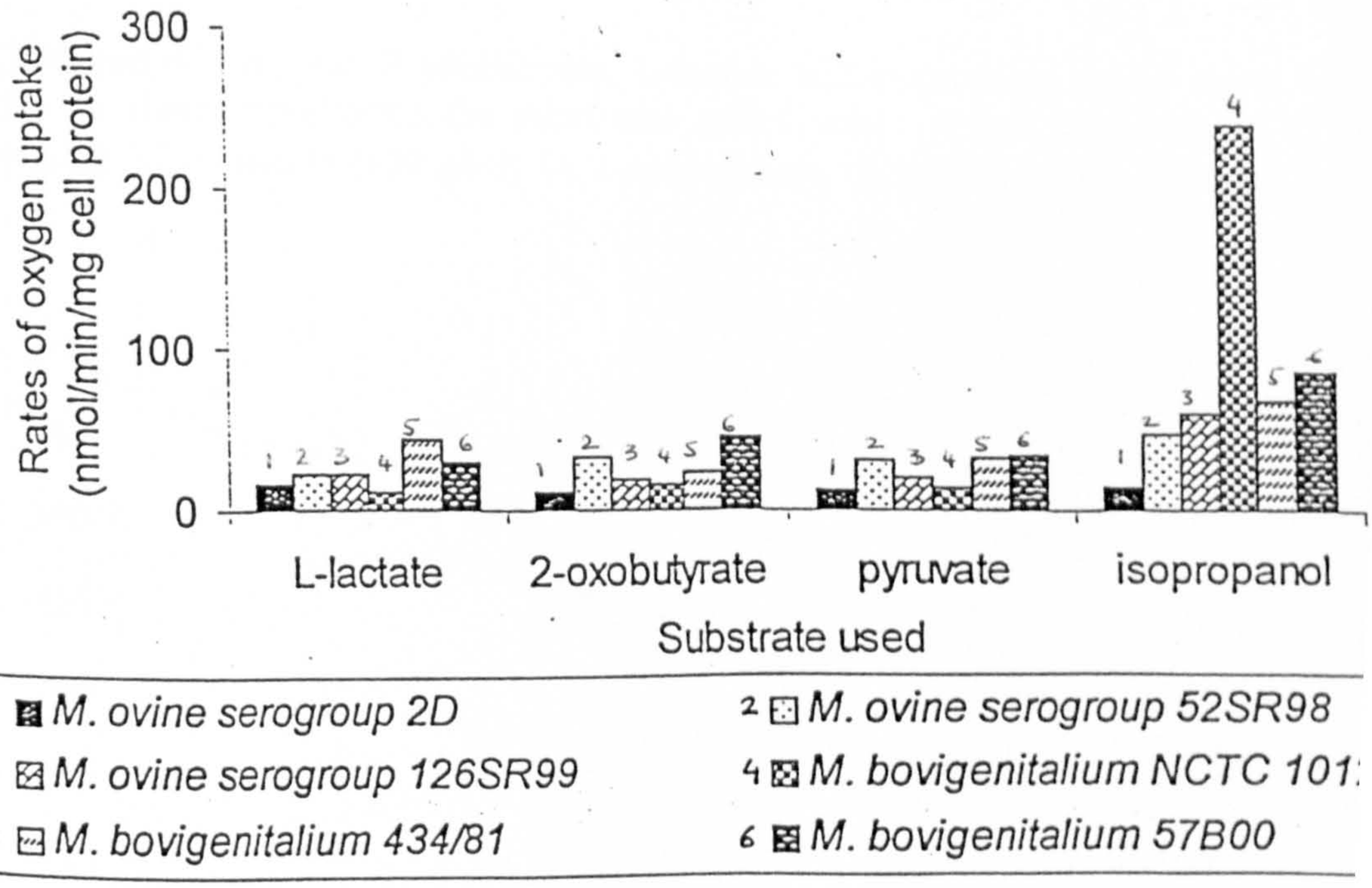


Figure 3.4 Oxidation of pyruvate, 2 oxobutyrate, L-lactate and isopropanol by *M. agalactiae* NCTC 10123. In these experiments the substrates added were: A (no substrate); B, pyruvate (100 μM); C, 2-oxobutyrate (100 μM); D, L-lactate, (100 μM); E, isopropanol (100 μM).

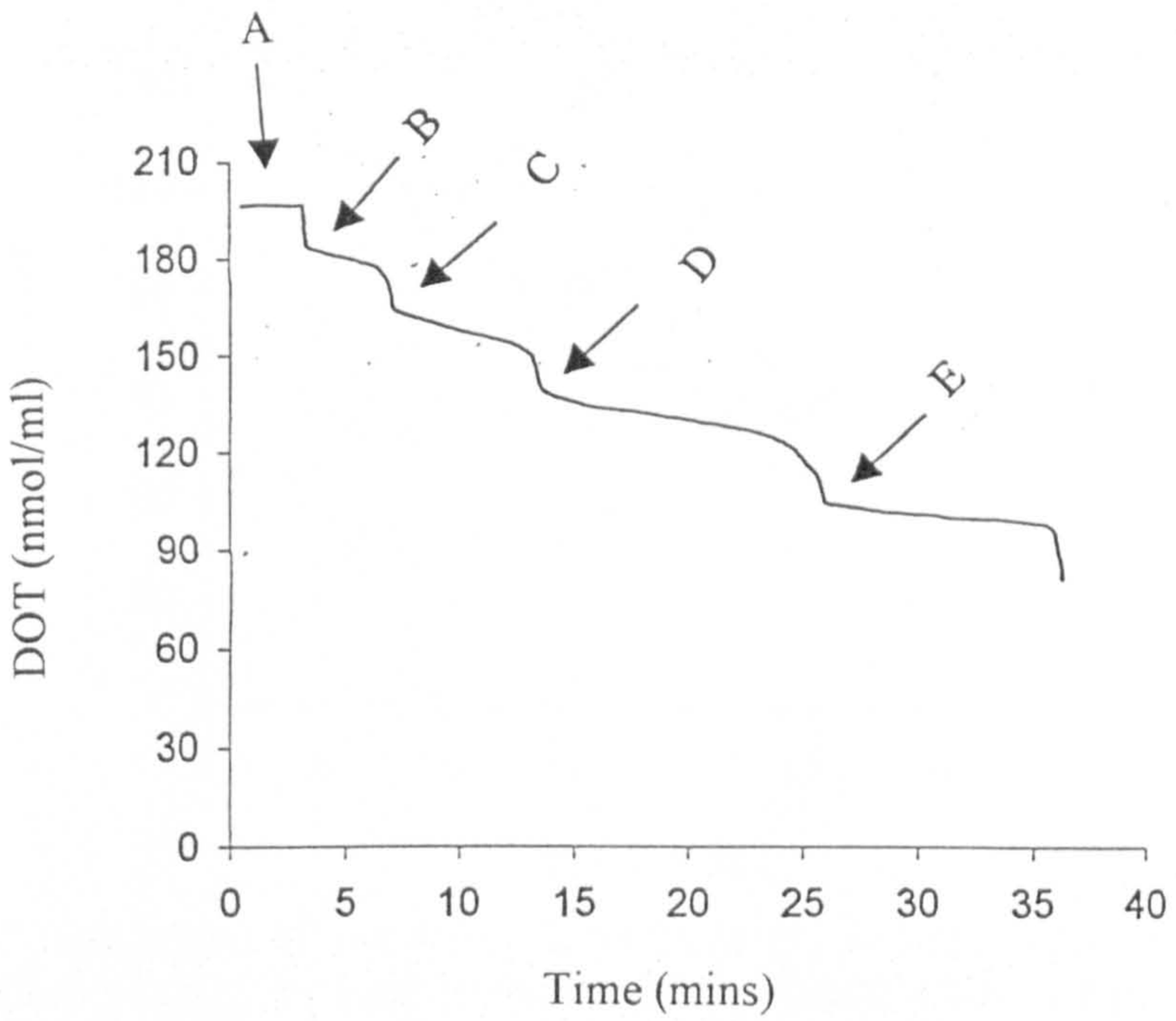


Figure 3.5 Oxidation of pyruvate, 2 oxobutyrate, L-lactate and isopropanol by *M. bovis* NCTC 10131. In these experiments the substrates added were: A (no substrate); B, pyruvate (100 μM); C, L-lactate (100 μM); D, 2-oxobutyrate, (100 μM); E, isopropanol (100 μM).

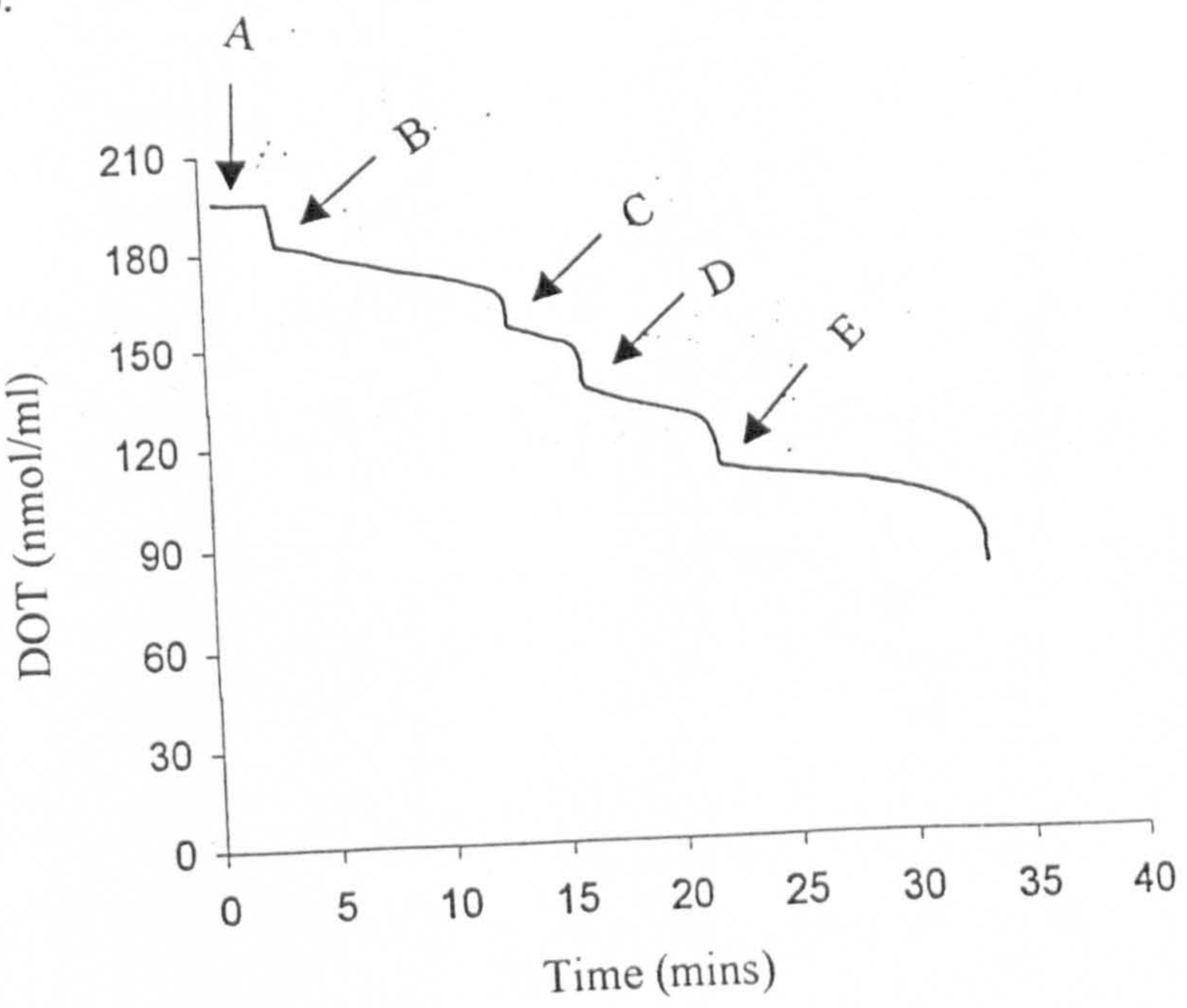


Figure 3.6 Oxidation of pyruvate, 2 oxobutyrate, L-lactate and isopropanol by *M.* ovine serogroup 11. In these experiments the substrates added were: A (no substrate); B, pyruvate (100 μM); C, 2-oxobutyrate (100 μM); D, L-lactate, (100 μM); E, isopropanol (100 μM).

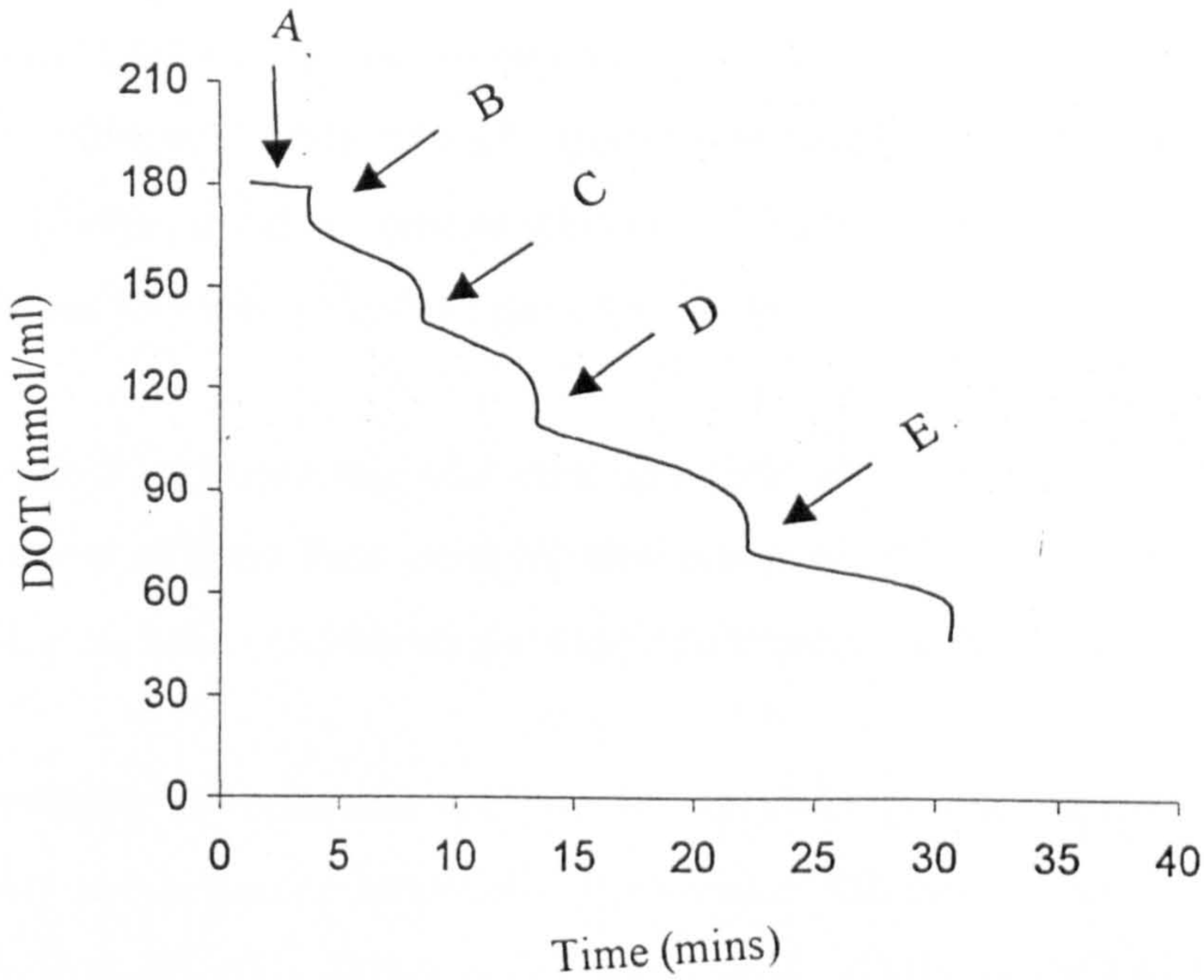
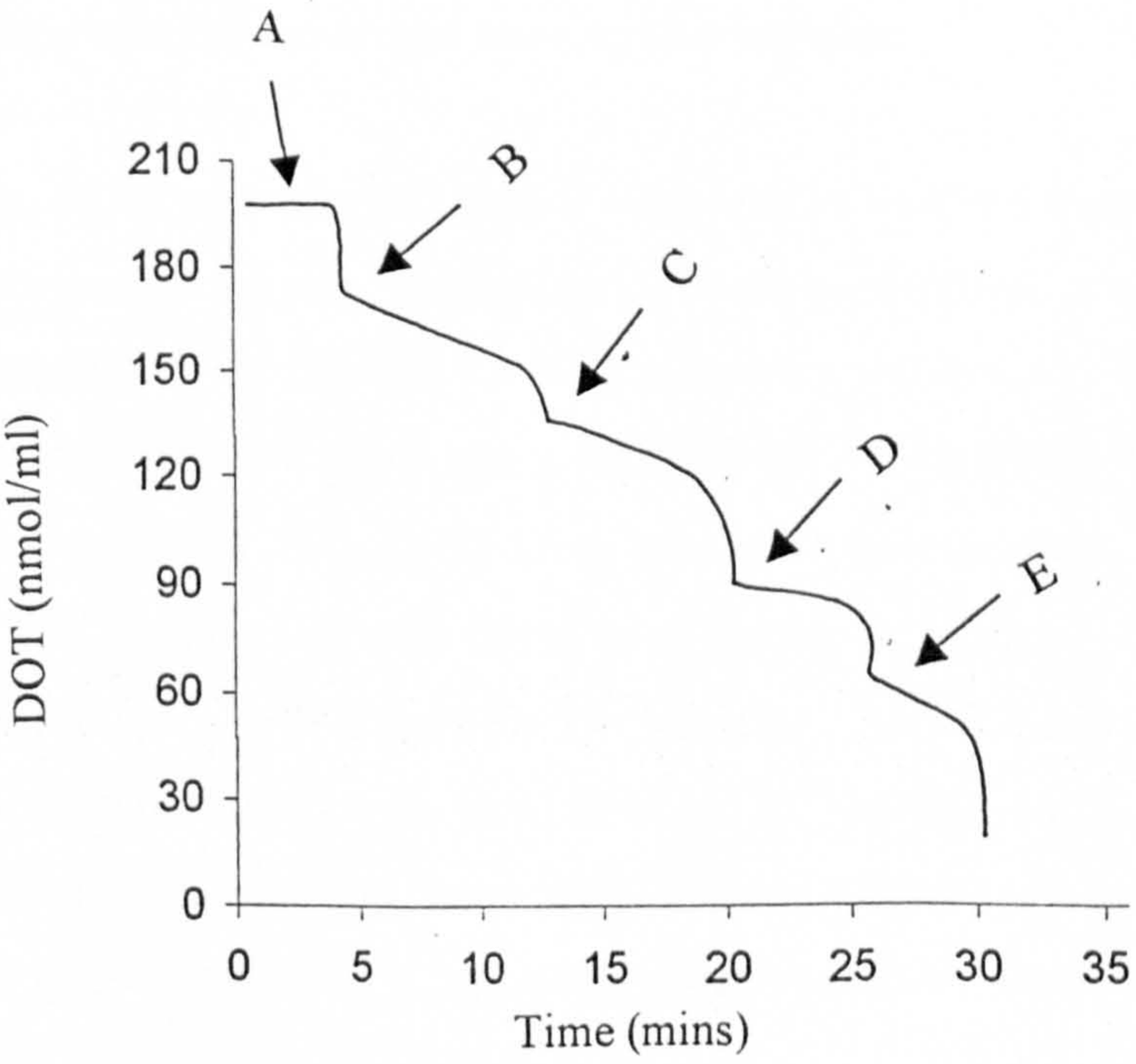


Figure 3.7 Oxidation of pyruvate, 2 oxobutyrate, L-lactate and isopropanol by *M. bovigenitalium* 434/81. In these experiments the substrates added were: A (no substrate); B, pyruvate (100 μM); C, 2-oxobutyrate (100 μM); D, L-lactate, (100 μM); E, isopropanol (100 μM).



Glucose was added (25 μM) to washed cell suspensions of *M. mycoides* SC. All strains were able to oxidise glucose at high rates with a high affinity for glucose. Rates of oxygen uptake were between 49-106 nmol/min/mg cell protein. The K_s values for metabolism of glucose were 2.5-3.6 μM, showing that there was a high affinity for this substrate (Table 3.9 and 3.11). Glucose oxidation by *M. mycoides* SC strain SH9 was quite different from the European strains. It did not oxidise glucose at 25 μM and oxidation at high concentration (250 μM) was very low 9 % of the rate of pyruvate.

The K_s value for glucose was also very high (965 μM) compared to other strains. This strain was very different from other SC strains studied. Glucose oxidation by LC was high and with low K_s values (3.58 μM) showing high affinity (Table 3.12).

Glucose-utilising mycoplasmas such as *M. mycoides* produce lactate anaerobically and acetate plus carbon dioxide aerobically (Rodwell and Mitchell, 1979). Glucose is present in bovine blood at 2-5 mM (Altman and Dittmer, 1974) and this might lead to sufficiently high concentrations on the mucous membranes of the respiratory tract for it to act as an effective energy source for all strains. These results were in agreement with Abu-Groun *et al.* (1994) who found similar patterns of substrate oxidation in *M. mycoides* strains.

3.3.2 Oxidation of fructose and N-acetylglucosamine

All strains of *M. mycoides* SC studied were able to oxidised fructose (25 μM). Relative rates of oxygen uptake were in the range of 59-84 % of the rate of glucose. The initial rates of oxygen uptake were between 41-59 nmol/min/mg cell protein. The K_s values were 2-3.2 μM, which showed that there was a high affinity for fructose. *M. mycoides* utilised N-acetylyglucosamine at 25 μM with initial rates of oxygen uptake of 19-36 nmol/min/mg cell protein. The relative rates were 30-55 % of the rate of glucose. The K_s values were <3 μ M, which showed a high affinity for this substrate (Table 3.9 and 3.10). Fructose and N-acetylglucosamine oxidation by strain SH9 and LC was high and K_s values were low. The relative rates of fructose and N-acetylglucosamine oxidation (% of pyruvate) were 131 % and 89 % respectively. The K_s values for these substrates were <4 μM. These results were

similar to the other SC strains. *M. mycoides* LC strain also oxidised fructose at a high rate 107 % (% of glucose) and K_s values were 4.38 μM. The rates of oxidation of N-acetylglucosamine (% of glucose) were 23 % and K_s values were 2.39 μM. It is possible, as there was a high affinity for N-acetylglucosamine and relatively low affinity for glucosamine, that glucosamine might not be used to a significant extent *in vivo*. These results were in agreement with Miles *et al*. (1985) found *M. mycoides* subsp. *mycoides* metabolised glucose, fructose, mannose, glucosamine, N-acetylglucosamine, glycerol, pyruvate and lactate. The result suggested, therefore that *M. mycoides* subsp. *mycoides* was adapted to, and was constitutive for, the utilisation of a single sugar (glucose) and a single amino sugar (N-acetylglucosamine), but that in the presence of fructose, a fructose-utilising pathway was induced. At birth, calves have a high level of blood fructose (approximately 3 mM), but this concentration declines rapidly to 0.15 mM within 24 hours (Young *et al*., 1970). In contrast glucose levels are high (2-5 mM) in foetal and adult cattle.

The ability to use sugars is generally constitutive though fructose metabolism in *M. mycoides* was four times greater in fructose-grown than in glucose-grown cells (Miles *et al*., 1985). Gaurivaud *et al*. (2000) have shown that fructose utilisation by the spiroplasmas in the phloem is linked to phytoplasma pathogenicity, so fructose utilisation in *M. mycoides* SC might be important in relation to its pathogenicity.

3.3.3 Oxidation of glucosamine and mannose.

All strains oxidised glucosamine and mannose 25μM at low rates compared to glucose. The relative rates of oxygen uptake (% of glucose) for glucosamine were 23-107 % and mannose 4-43 % (Table 3.10). The initial rates of oxygen uptake for glucosamine and mannose were 12-44 and 4-20 nmol/min/mg cell protein (Table 3.9). The K_s values were 0.8-4.3 μM for glucosamine and 156-457 μM for mannose (Table 3.11) showing a low affinity for mannose. The glucosamine K_s values were low for all the strains showing high affinity for the substrate. When the substrate concentration (glucosamine and mannose) was increased the rate of substrate oxidation increased. The relative rates of oxygen uptake (% of glucose) for mannose at 250 μM were 16-92 %. The relative rates of oxidation by strain SH9 for glucosamine and mannose (% of pyruvate) were 11 and 15 % at 250 μM. This

strain (SH9) was different from other European SC strains as it oxidised glucosamine at low rate although mannose was oxidised at same rate. The K_s values for these substrates were 2.2 mM for glucosamine and 618 μM for mannose. The relative rates of oxidation by LC strain were for (% of glucose) 42 % for glucosamine and 36 % for mannose. The K_s value was high for glucosamine (818 μM) but low for mannose (4 μM) (Table 3.12). The ability to utilise N-acetylglucosamine and mannose is of particular interest since, although they are major constituents of mammalian glycoconjugates, they normally occur in body fluids as free sugars only at very low concentrations. However, host derived N-acetylglucosaminidase and mannosidase activities (principally from lysosomes) may be released into cell surroundings following cellular damage. The ability to use sugars such as glucosamine and mannose might reflect the pathogenicity of these strains.

3.3.4 Oxidation of maltose and trehalose.

All *M. mycoides* SC strains were unable to utilise maltose and trehalose at higher concentration (2.5 mM) while *M. mycoides* LC did oxidise maltose. The relative rates of oxidation (% of glucose) for maltose were 54 % and the K_s values were low (1.71 μM) showing the high affinity for this substrate (Table 3.12). These results were in agreement with that of Abu-Groun *et al.* (1994) who showed that the disaccharides maltose and trehalose were not oxidised by *M. mycoides*. Glucosidase activity is absent in *M. mycoides* subsp. *mycoides* SC strain T_1; this activity is required for the oxidation of maltose. However maltose was utilised by *M. mycoides* subsp. *capri* (Wadher and Miles, 1988).

3.3.5 Oxidation of glycerol

All these European strains were unable to oxidise glycerol at higher concentration (2.5 mM). SH9 strain, which was isolated from Africa, oxidised glycerol and the relative rates (% of pyruvate) of oxidation was 349 %. The K_s value was low (5.1 μM). The relative rate of glycerol oxidation by LC was 292 % and K_s value was low (5.88 μM). These results were in agreement with Houshaymi *et al.* (1997) who reported that European strains were unable to oxidise glycerol, which distinguished them from African strains. However Abu-Groun *et al.* (1994) found all African and Australian *M. mycoides* SC strains oxidised

glycerol. Glycerol oxidation might be a useful marker in epidemiological studies of these strains.

3.3.6 Oxidation of organic acids

All strains were able to oxidise pyruvate, lactate and 2-oxobutyrate at 50 μM. The initial rates of oxygen uptake were for L-lactate 22-82, pyruvate 9-48 and 10-81 nmol/min/mg cell protein. The relative rates of oxygen uptake (% of glucose) were for L-lactate, 23-169 % pyruvate, 11-69 % and 2-oxobutyrate, 12-99 %. *M. mycoides* SC Portugal clone 6 strain oxidised organic acids at higher rates compared to the other small colony clones. (Table 3.10).

The K_s values for the organic acids metabolism were for L-lactate, 5.3-27μM, and pyruvate 7.2-16.8 μM and 2-oxobutyrate, 8.5-25μM (Table 3.11). The organic acids oxidation by strain SH9 was high with relative rates of oxygen uptake (% of pyruvate) L-lactate, 228 % and 2-oxobutyrate, 99 %. The K_s value were also low showing high affinity for these substrates. The K_s values for L-lactate were 9 μM, pyruvate, 10 μM and 2-oxobutyrate, 8.7 μM. The organic acids oxidation by LC was also high with relative rates of oxygen uptake (% of glucose) L-lactate 164 %, pyruvate 72 % and 2-oxobutyrate 64 %.

The K_s values for L-lactate, 7.3 μM, pyruvate, 6.3 μM and 2-oxobutyrate 6 μM (Table 3.12). The oxidation of pyruvate and lactate require a common component for transport in *M. mycoides* subsp. *mycoides* strain T_1 (Lee *et al.*, 1986). Pyruvate may be used as an energy source for the *M. mycoides* subsp. *capri* (Miles *et al.*, 1988) and the concentration of lactate and pyruvate in body fluids is sufficiently high (e.g. 0.5 and 0.05 mM respectively in bovine serum; Altman and Dittmer, 1974) to suggest that strains are adapted to utilise these substrates *in vivo*. The ability to utilise lactate at high rates has also been proposed as a pathogenicity factor in *Neisseria gonorrhoea* (Britigan *et al.*, 1988) and 2-oxobutyrate, an intermediate in threonine degradation (Rodwell, 1960) was also oxidised at a high rate by all SC strains. It is suggested that pyruvate and 2-oxobutyrate might be a substrate of the same uptake system because of their structural similarity. In *M. mycoides,* pyruvate may be metabolised to acetate in a series reaction involving pyruvate dehydrogenase, phosphate

acetyl transferase and acetate kinase and high acetate kinase activities have been demonstrated in *Acholeplasma* and *Mycoplasma* species (Kahane *et al,* 1978).

3.4 The effect of metabolisable substrates on the growth of *M. agalactiae, M. bovis, M. bovigenitalium* and *M.* ovine serogroup 11

Miles *et al.* (1994) reported that mycoplasmas obtain their energy from the fermentation of sugars, the hydrolysis of arginine, the oxidation of certain organic acids or any combination of these substrates. They also showed variation in the pattern of oxidation of these substrates. The aim of the current work was to identify the most suitable organic acid and its optimum concentration, for the growth of *M. agalactiae*, *M. bovis, M. bovigenitalium* and *M.* ovine serogroup11. Another aim of the study was to modify PRM medium by replacing ruminant sources of peptones by a vegetable source.

Alcohols, especially isopropanol, were oxidised at very high rates by all strains of these species. Isopropanol was also added to the medium and its effect on growth of these organisms was monitored. Different growth media were also tested for the growth of *M. bovis.* A variety of media have been used for the growth of these species. These differ in the nature, source and concentrations of the components used. To maximize cells yield and growth rate, the role of different potential substrates was determined.

M. agalactiae type strain NCTC 10123, field strain 453/94, *M. bovis* type strain NCTC 10131, field strain 137B99, *M. bovigenitalium* type strain NCTC 10122 field strain 398/87 and *M.* ovine serogroup 11 strain 2D, field strains 3SR99 and 48SR99 were grown in the presence of organic acids (L-lactate, pyruvate and 2-oxobutyrate) at different concentrations (0, 0.2, 0.5 and 1.0 % w/v). For all strains the addition of pyruvate and 2-oxobutyrate 0.2-0.5 % (w/v) increased the growth rate of these strains. The maximum growth rate was obtained by the addition of 0.5 % (w/v) pyruvate and 2-oxobutyrate to the medium.

Table 3.9 Biochemical characteristics of *M. mycoides* SC. Initial rates of oxygen uptake (nmol/min/mg cell protein).

Substrate	**Substrate conc. (μM)**	**Strain code (clone)**					
		1	2	3	4	5	6
Glucose	25	64	79	49	106	83	82
Fructose	25	44	42	41	55	59	52
Glucosamine	25	18	21	12	44	19	22
GlcNAG	25	28	27	19	36	26	27
Mannose	25	9	5	4	4	5	20
Maltose	2.5 mM	Nd	Nd	Nd	Nd	Nd	Nd
Trehalose	2.5 mM	Nd	Nd	Nd	Nd	Nd	Nd
Pyruvate	100	16	9	20	32	43	48
L-lactate	100	34	25	22	37	44	82
2-oxobutyrate	100	21	10	16	25	15	81
Glycerol	2.5 mM	Nd	Nd	Nd	Nd	Nd	Nd

Table 3.10 Biochemical characteristics of *M. mycoides* SC. Relative rates of oxygen uptake (% of glucose).

Substrate	**Substrate conc. (μM)**	**Strain code (clone)**					
		1	2	3	4	5	6
Glucose	25	100	100	100	100	100	100
Fructose	25	78	59	84	62	68	63
Glucosamine	25	29	24	23	38	32	107
GlcNAG	25	43	45	39	55	30	46
Mannose	25	12	8	9	4	5	43
Maltose	2.5 mM	Nd	Nd	Nd	Nd	Nd	Nd
Trehalose	2.5 mM	Nd	Nd	Nd	Nd	Nd	Nd
Pyruvate	100	26	11	21	29	54	69
L-lactate	100	44	29	23	34	56	169
2-oxobutyrate	100	28	12	16	23	19	99
Glycerol	2.5 mM	Nd	Nd	Nd	Nd	Nd	Nd

Nd, not metabolised.

Table 3.11 Biochemical characteristics of *M. mycoides* SC. K_s values (μM).

Substrate	Strain code (clone)					
	1	2	3	4	5	6
Glucose	2.5	3	3.6	3.5	2.7	3.2
Fructose	2	2	3	2.3	3.2	2.9
Glucosamine	1.6	2.5	2	0.8	4.3	0.9
GlcNAG	1.6	1.8	2.5	1.7	2.8	2
Mannose	156	457	192	236	202	123
Maltose	Nd	Nd	Nd	Nd	Nd	Nd
Trehalose	Nd	Nd	Nd	Nd	Nd	Nd
Pyruvate	22	17	8	7.2	13	12
L-lactate	8.6	27	11.5	5.8	5.3	7.9
2-oxobutyrate	12.8	25.2	9.5	8.5	8.6	17.5
Glycerol	Nd	Nd	Nd	Nd	Nd	Nd

Nd, not metabolised

Table3.12 Biochemical characteristics of *M. mycoides* SC strain SH9 and *M. mycoides* LC.

Substrate	**Concentration (μM)**	***M. mycoides* SC SH9 Relative rates of oxygen uptake (% of pyruvate)**	**SH9 *K*,values (μM)**	***M. mycoides* LCRelative rates of oxygen uptake (% of glucose)**	**L C *K*, values (μM)**
Glucose	25 250 2.5 mM	Nd 9 20	965	100	3.58
Fructose	25	131	3.87	107	4.38
Glucosamine	250 2.5mM	11 39	2.2mM	42 129	818
N-acetyl-glucosamine	25	89	3.65	23	2.9
Mannose	250 2.5mM	15 55	618	36	4
Maltose	25 2.5mM	Nd	Nd	54	1.71
Trehalose	2.5mM	Nd	Nd	Nd	Nd
Pyruvate	100	100	10.5	72	6.27
L-lactate	100	228	9.12	164	7.3
2-oxobutyrate	100	99	8.72	64	5.91
Glycerol	50	349	5.1	292	5.88

Nd, not metabolised

In contrast the addition of L-lactate had little effect on the growth of these species. As lactate is first oxidised to pyruvate by lactate dehydrogenase, it is possible that growth of these organisms in the presence of lactate may be increased by increasing aeration.

Isopropanol was also supplemented in the basal PRM medium 0, 0.2, 0.5 and 1.0 % (v/v) and it was observed to inhibit the growth of all strains tested (Figure 3.8, 3.9, 3.10, 3.11, 3.12, 3.13, 3.14, 3.15 and 3.16). This indicates that isopropanol is not a source of energy hence its oxidation at very high rate by all the strains is unclear. It is possible that the concentration used for isopropanol was equal to organic acids, which might be toxic to cells and hence may have caused inhibition of growth.

M. bovis type strain NCTC 10131 was grown in four different media (SP4, PPM, PRM and Eaton's media) supplemented with two concentrations of pyruvate and glucose (0.2 and 0.5 % w/v). The aim of this work was to identify the best medium for the growth of *M. bovis*, including optimum pyruvate concentration and the effect of glucose on the growth of *M. bovis*. The effect of pyruvate and glucose was monitored by measuring the culture absorbance, protein concentration and pH change after every 24 hours. It was observed in all cases that addition of pyruvate increased the growth yield compared to control (without addition of pyruvate). The growth yield was maximum when pyruvate was added at 0.5 % (w/v) in all the tested media. The addition of glucose to all the media did not cause any effect. The maximum absorbance recorded in the presence of 0.5 % (w/v) pyruvate was in SP4 medium (0.184), PPM (0.299), PRM (0.229) and Eaton 's medium (0.308). It was observed that the growth of *M. bovis* type strain in Eaton's medium was maximum when supplemented with (0.5 % w/v) pyruvate. The growth of *M. bovis* in SP4 and PRM media was less and in PPM medium growth rate was high at 0.5% (w/v) pyruvate. The growth of these organisms was not enhanced in the presence of pyruvate in SP4 medium, which is a very enriched medium. Growth of *M. bovis* was very slight in control media in all cases and the maximum absorbance was 0.05 (Figure 3. 17, 3.18, 3.19 and 3.20).

The rate of change of pH is proportional to the rate of substrate metabolism (Table 3.13). This need not be the case as pH change will be affected by the dissociation constant pka values of acidic and basic groups present and in any case pH is logarithmically (and not

linearly) related to hydrogen ion concentration. Some non-fermenting organisms may show a change in pH, which is produced by the metabolism of substrates other than glucose. Growth media for mycoplasmas contain serum and yeast extract a slight fall in pH may also occur even in uninoculated media after several days of incubation. This may be avoided by the use of media without yeast extract. The pH of medium is an important factor which affects bacterial viability (Gourlay and Macleod, 1966). Windsor (1978) showed that it was drop in pH and not a loss of nutrients during growth that was the cause of bacterial death following entry into stationary phase.

The growth of *M. bovis* was maximum in Eaton's medium when supplemented with pyruvate. This medium was further optimised by using different concentrations of pyruvate. *M. agalactiae* and *M. bovis* field strains (101/94 and 82B96 respectively) were grown in Eaton's medium supplemented with increasing concentrations of pyruvate: 0, 0.2, 0.4, 0.5, 0.6, 0.8 and 1.0 % (w/v). Culture absorbance, pH and protein concentration were measured at 24 hour intervals. It was observed that increasing concentrations of pyruvate increased cell yields but 1.0 % (w/v) pyruvate inhibited the growth of these organisms (Figure 3.21 and 3.22). Growth of these organisms in Eaton's medium without pyruvate was not enhanced and maximum absorbance obtained was 0.078. The ability of pyruvate to act as a source of energy may be of particular relevance to the *in vitro* cultivation of strains which fail to ferment glucose or hydrolyse arginine and which grow poorly in conventional mycoplasma media e.g. the medium for *M.* F38 contained *viande foie* digest (animal tissue) and 50 % (v/v) goat serum (Cottew, 1983).

So pyruvate acts as a source of energy and carbon source for non-fermentative and non-arginine-hydrolysing mycoplasmas. Growth of these organisms was increased by the addition of pyruvate in Proteose Peptone (PP) medium. These species did not oxidise glucose nor was it a source of energy. Pyruvate may be utilised in the absence of reaction involving molecular oxygen; oxidation of pyruvate to acetate plus CO_2 may be balanced by the reduction of an equal quantity of pyruvate to lactate (Walker, 1959). *M. gallinarium*, which does not ferment sugars, but hydrolyses arginine, effectively oxidised the organic acids pyruvate, lactate and 2-oxobutyrate which serve as a source of energy (Taylor *et al.*, 1994). *M. agalactiae* and *M. bovis* have shown maximum growth when pyruvate was

added at 0.5 % (w/v). This concentration can be used in the medium for the growth of these species. This study provides data, which can be used to develop an improved and selective growth medium for the growth of *M. agalactiae, M. bovis, M. bovigenitalium and M.* ovine serogroup 11, which could potentially serve as a veterinary aid in the diagnosis of diseases caused by these organisms.

These results agreed with the study of Miles *et al.* (1988) who reported that the growth of certain non-fermentative and non-arginine hydrolysing *Mycoplasma* species increased when pyruvate was added to the growth medium. Growth in the presence of lactate was not enhanced. In anaerobic conditions one mol of pyruvate is reduced to lactate and one mol of lactate is oxidised to produce acetate, CO_2 and ATP. It has been suggested that 2-oxobutyrate may be oxidised to propionic acid and reduced to 2-hydroxybutyric acid. Pyruvate has the same pathway and growth was similar when pyruvate and 2-oxobutyrate was supplemented in the medium.

PRM medium was modified for growth of *M. bovis.* This medium which was formulated for the growth of *M. mycoides* SC and contains special peptone as a source of polypeptides and amino acids with pyruvate at 0.2 % (w/v) was used as an energy source. The growth of *M. bovis* can be enhanced by increasing pyruvate to 0.5 % (w/v) in PRM medium. Vegetable peptones have not been used so far in mycoplasma growth media and they are important source of polypeptides. Mycoplasmal media contain ruminant sources of peptones, which are risky to use for the growth of vaccine strains. There may be a risk of spreading bovine spongiform encephalopathy (BSE) by using ruminants sources of peptones. Replacing ruminant source peptones with vegetable peptones is very advantageous as risks of spreading of BSE can be diminished. Different vegetable peptones such as vegetable peptone broth, vegetable peptone 1, tryptone soya broth, neutralised soya broth and PPLO broth were tested for the growing *M. bovis* vaccine strain 86B96. Three different concentrations (7, 15 and 20 g/l) were used. Growth was monitored by measuring OD at 540 nm and by viable count. *M. bovis* strain 86B96 gave high growth with all peptones after 24 hours. The optimal concentration of peptones was determined. Comparatively highest yields and growth rates were obtained when vegetable peptone broth 15g/l was added to the medium (Figure 3.23). All other peptones also gave good yield and 10^9 cfu/ml was obtained after 24 hours incubation.

Table 3.13 Change of pH during growth of *M. bovis* strain NCTC 10131 in different media. Initial pH of the media was 7.6.

Medium	**PH change (units)**	**Medium**	**pH change (units)**
SP4 0.2 % pyruvate	7.4 (0.2)	**PRM** 0.2 % pyruvate	7.5 (0.1)
SP4 0.5 % pyruvate	7.3 (0.3)	**PRM** 0.5 % pyruvate	6.8 (0.8)
SP4 0.2 % pyruvate and 50 % glucose	7.5 (0.1)	**PRM** 0.2 % pyruvate and 0.5 % glucose	6.9 (0.7)
SP4 0.5 % pyruvate and 50 % glucose	7.3 (0.3)	**PRM** 0.5 % pyruvate and 0.5 % glucose	6.8 (0.8)
SP4 control	7.5 (0.1)	**PRM** control	7.2 (0.4)
PP medium 0.2% pyruvate	6.6 (1.0)	**Eaton** 's medium 0.2 % Pyruvate	7.2 (0.4)
PP medium 0.5 % pyruvate	6.4 (1.2)	**Eaton** 's medium 0.5 % pyruvate	7.00 (0.6)
PP medium 0.2 % pyruvate and 0.5 % glucose	6.6 (1.0)	**Eaton**'s medium 0.2 % pyruvate and 0.5% glucose	7.3 (0.3)
PP medium 0.5 % pyruvate and 0.5 % glucose	6.4 (1.2)	**Eaton** 's medium 0.5 % pyruvate and 0.5 % glucose	7.00 (0.6)
PP medium control	6.8 (0.8)	**Eaton**'s media control	7.5 (0.1)

Figure 3.8 The effect of organic acids and isopropanol on the growth of *M. bovis* type strain NCTC 10131. Growth was measured as increase in OD (540 nm) and determined at 24, 48 and 72 hours. Data shown are maximum values, which in all cases were reached at either 24 or 48 hours. Data are means ± SD of three independent experiments.

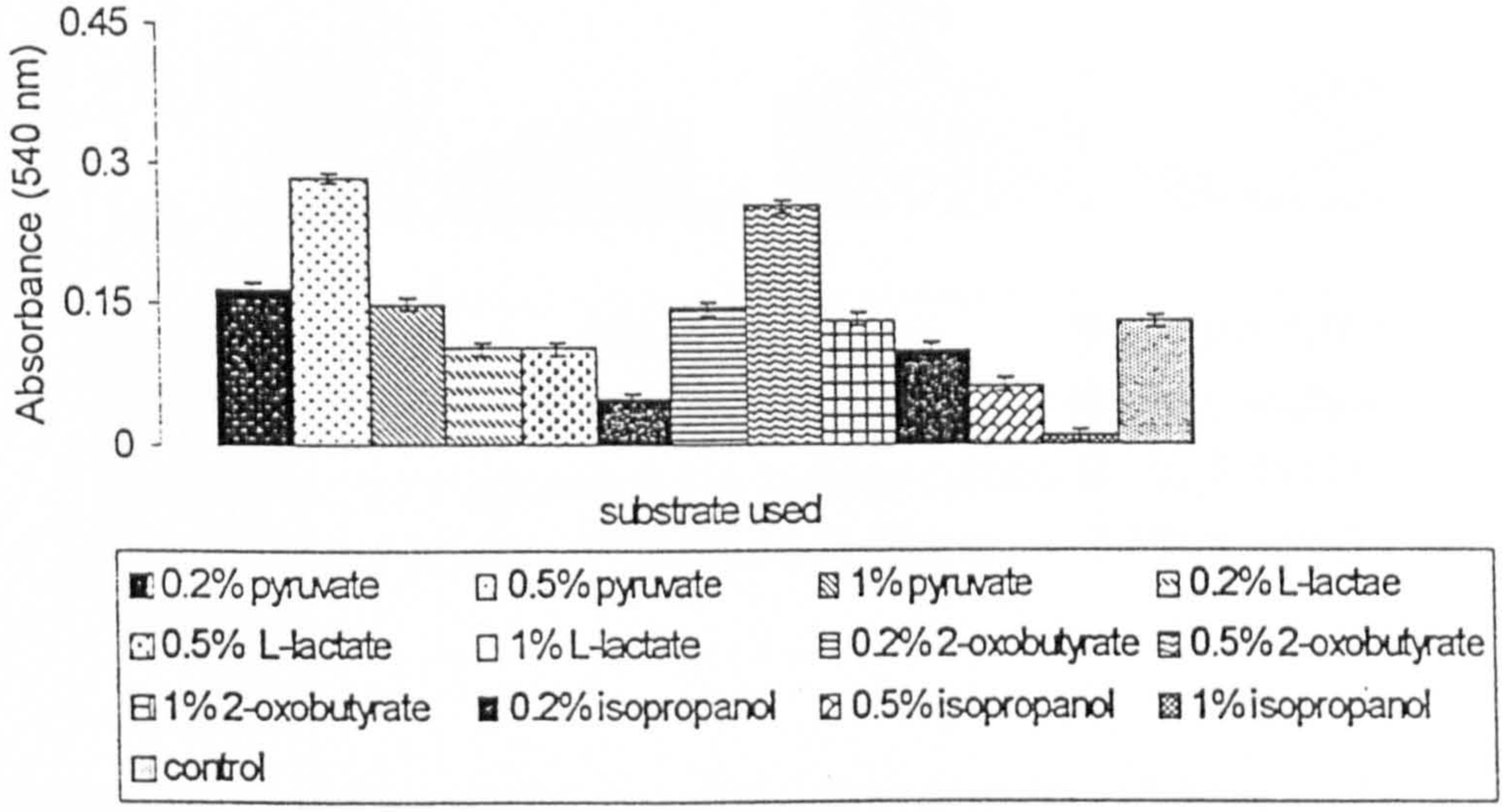


Figure 3.9 The effect of organic acids and isopropanol on the growth of *M. bovis* 137B99. Growth was measured as increase in OD (540 nm) and determined at 24, 48 and 72 hours. Data shown are maximum values, which in all cases were reached at either 24 or 48 hours. Data are means ± SD of three independent experiments.

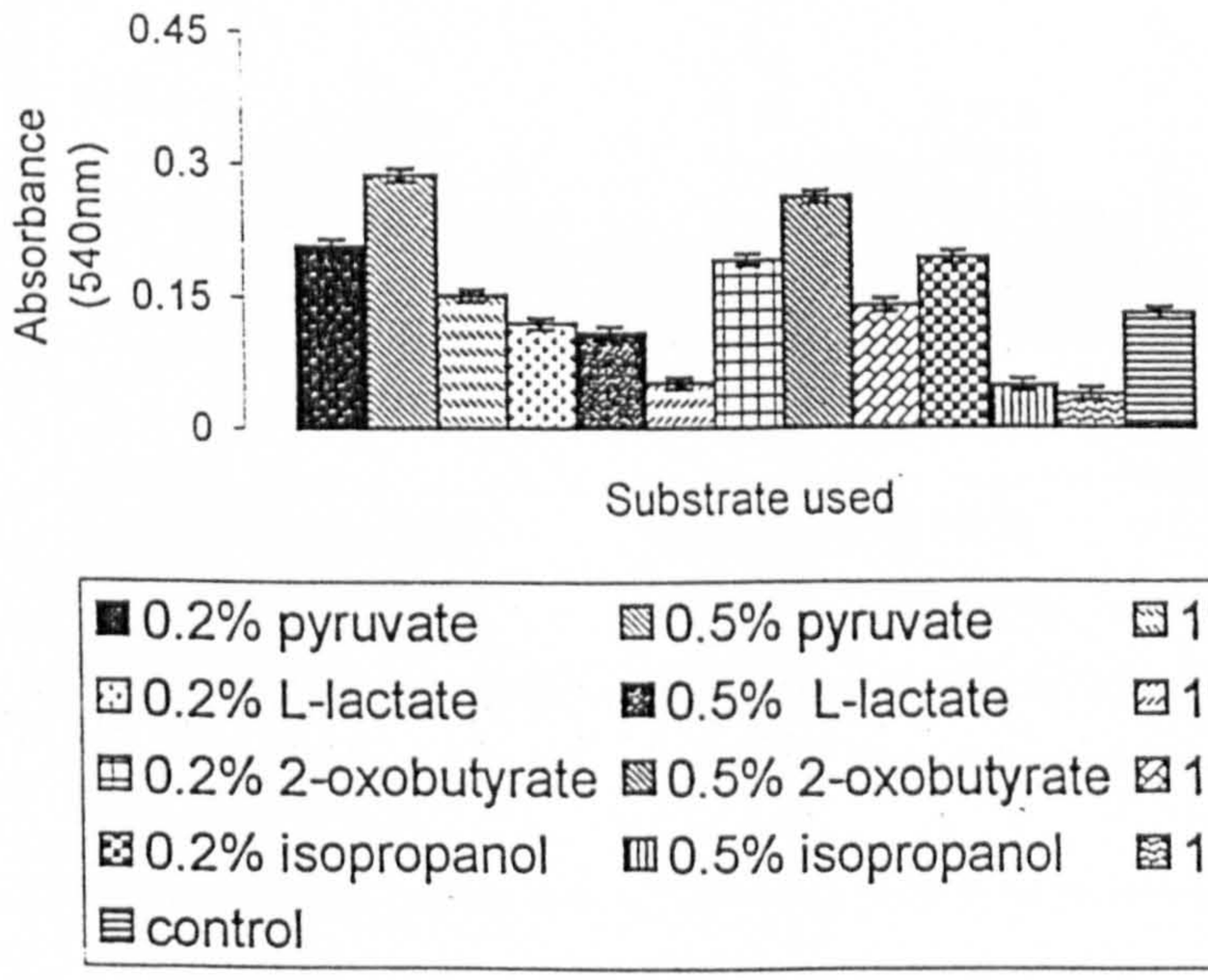


Figure 3.10 The effect of organic acids and isopropanol on the growth of *M. agalactiae* type strain NCTC 10123. Growth was measured as increase in OD (540 nm) and determined at 24, 48 and 72 hours. Data shown are maximum values, which in all cases were reached at either 24 or 48 hours.

Data are means ± SD of three independent experiments.

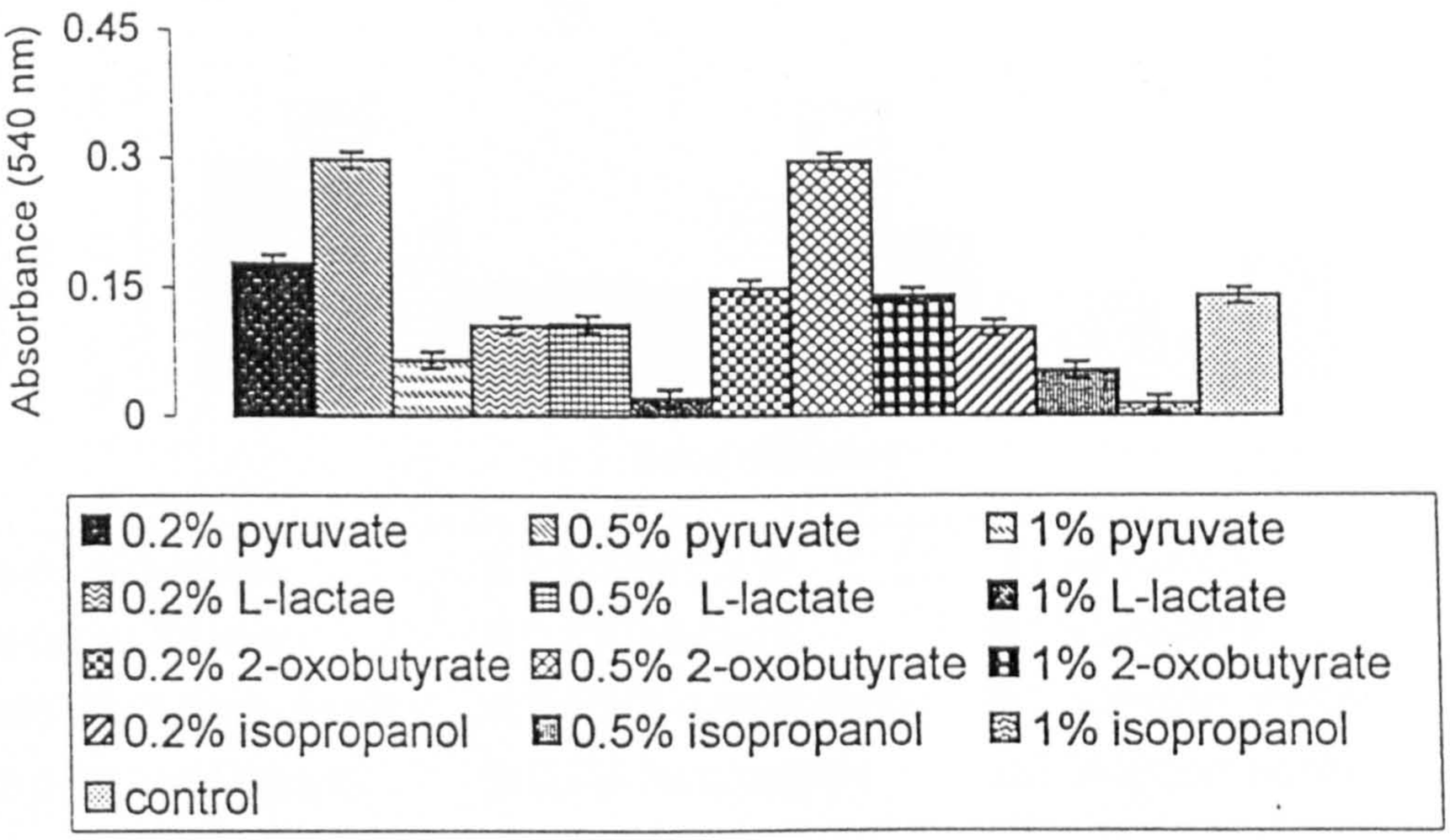


Figure 3.11 The effect of organic acids and isopropanol on the growth of *M. agalactiae* 453/94. Growth was measured as increased in OD (540 nm) and determined at 24, 48 and 72 hours. Data shown are maximum values, which in all cases were reached at either 24 or 48 hours. Data are means ± SD of three independent experiments.

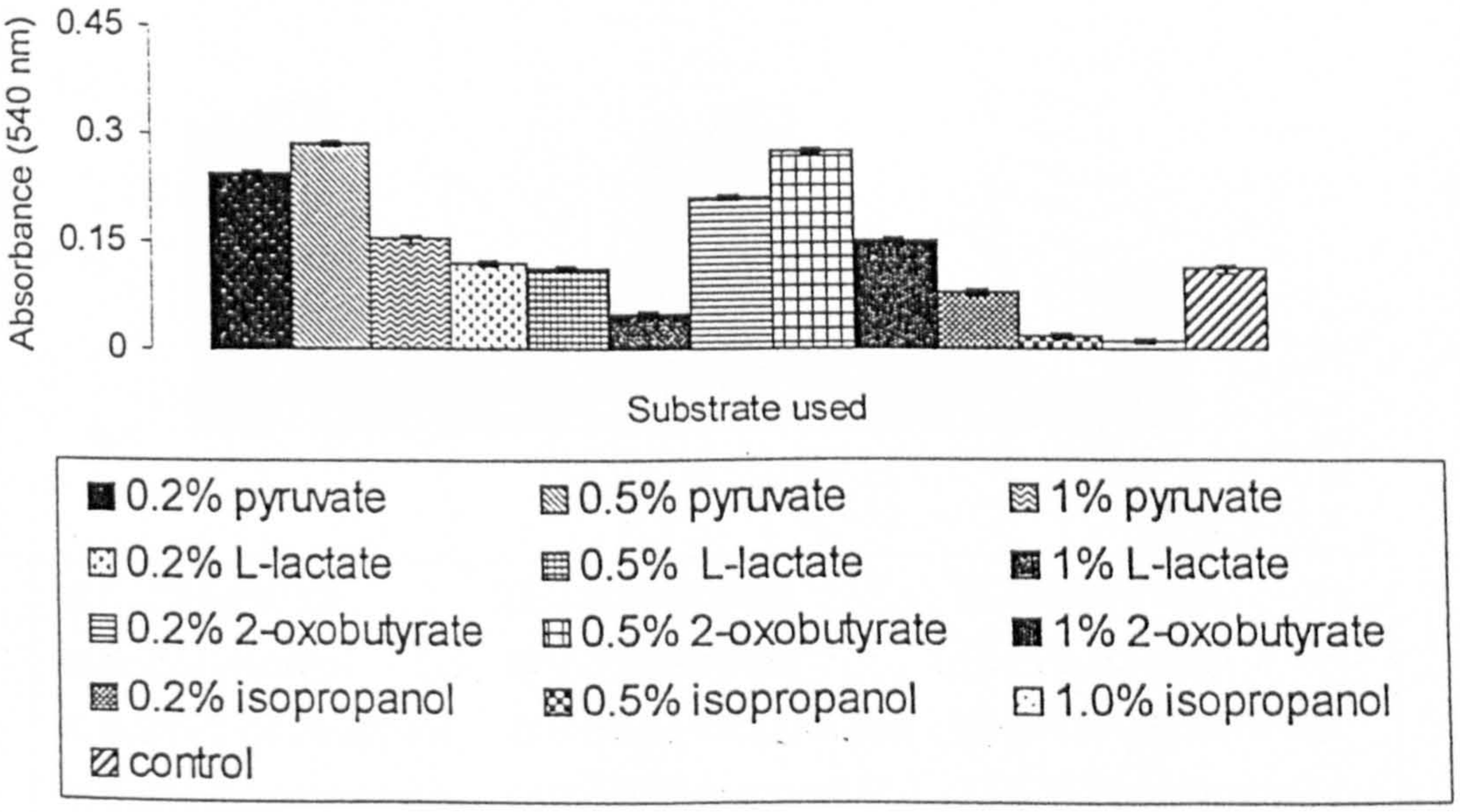


Figure 3.12 The effect of organic acids and isopropanol on the growth of *M.* ovine serogroup 11 strain 2D. Growth was measured as increase in OD (540 nm) and determined at 24, 48 and 72 hours. Data shown are maximum values, which in all cases were reached at either 24 or 48 hours. Data are means ± SD of three independent experiments.

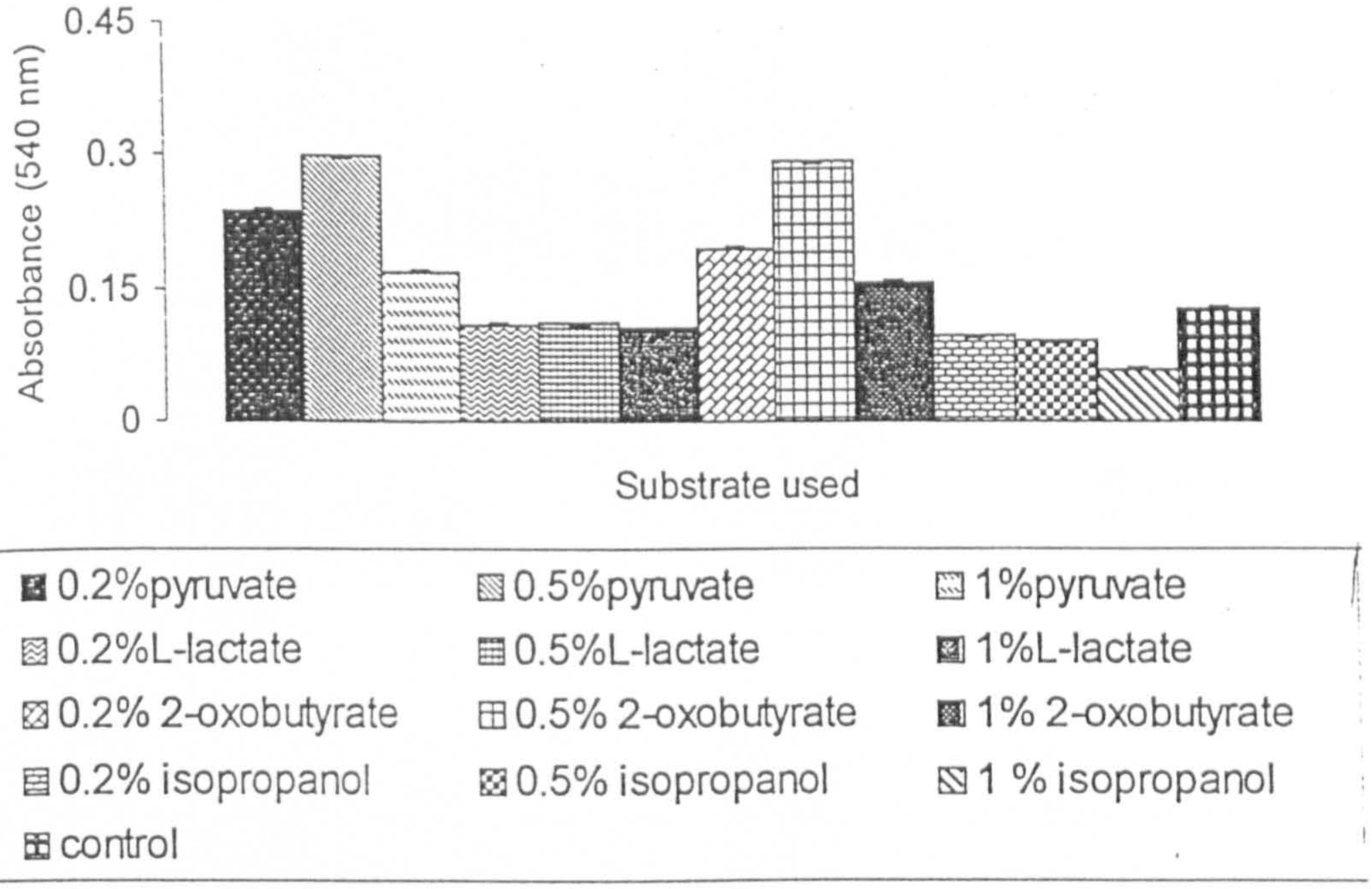


Figure 3.13 The effect of organic acids and isopropanol on the growth of *M.* ovine serogroup 11strain 3SR99. Growth was measure as increase in OD (540 nm) and determined at 24, 48 and 72 hours. Data shown are maximum values, which in all cases were reached at either 24 or 48 hours.

Data are means ± SD of three independent experiments.

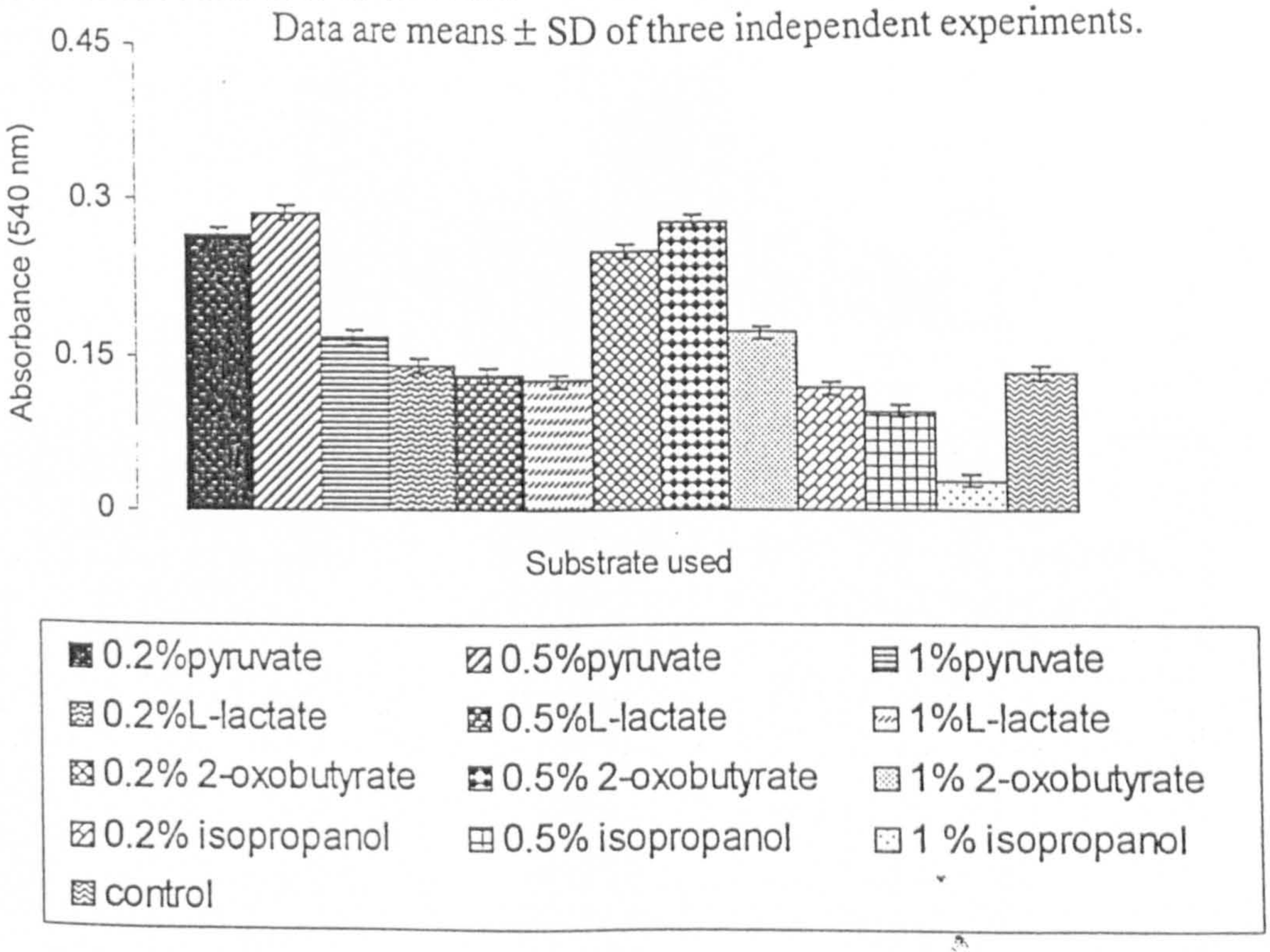


Figure 3.14 The effect of organic acids and isopropanol on the growth of *M.* ovine serogroup 11strain 48SR98. Growth was measured as increased in OD (540 nm) and determined at 24, 48 and 72 hours. Data shown are maximum values, which in all cases were reached at either 24 or 48 hours.

Data are means ± SD of three independent experiments.

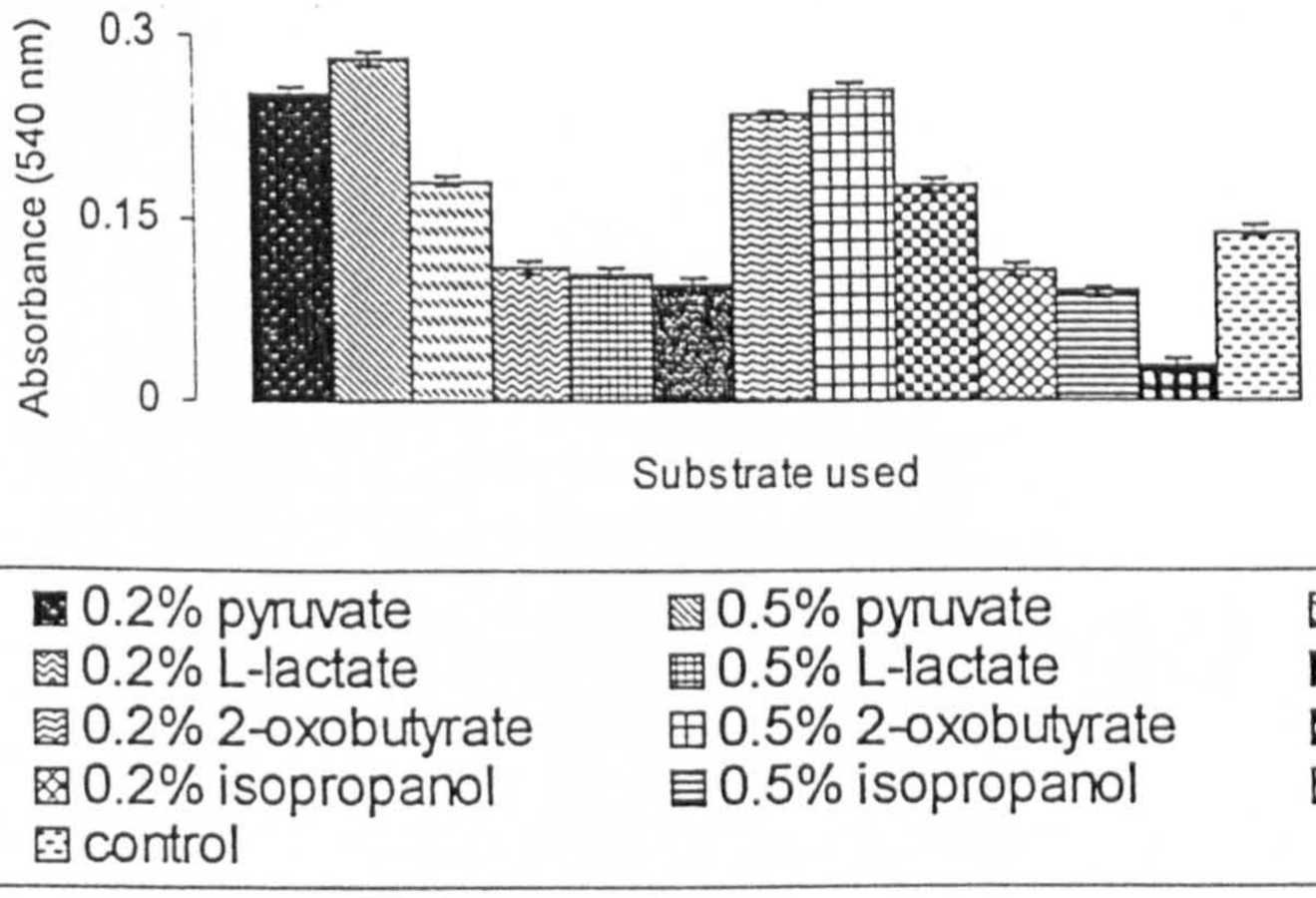


Figure 3.15 The effect of organic acids and isopropanol on the growth of *M. bovigenitalium* type strain NCTC 10122. Growth was measured as increase in OD (540 nm) and determined at 24, 48 and 72 hours. Data shown are maximum values, which in all cases were reached at either 24 or 48 hours.

Data are means ± SD of three independent experiments.

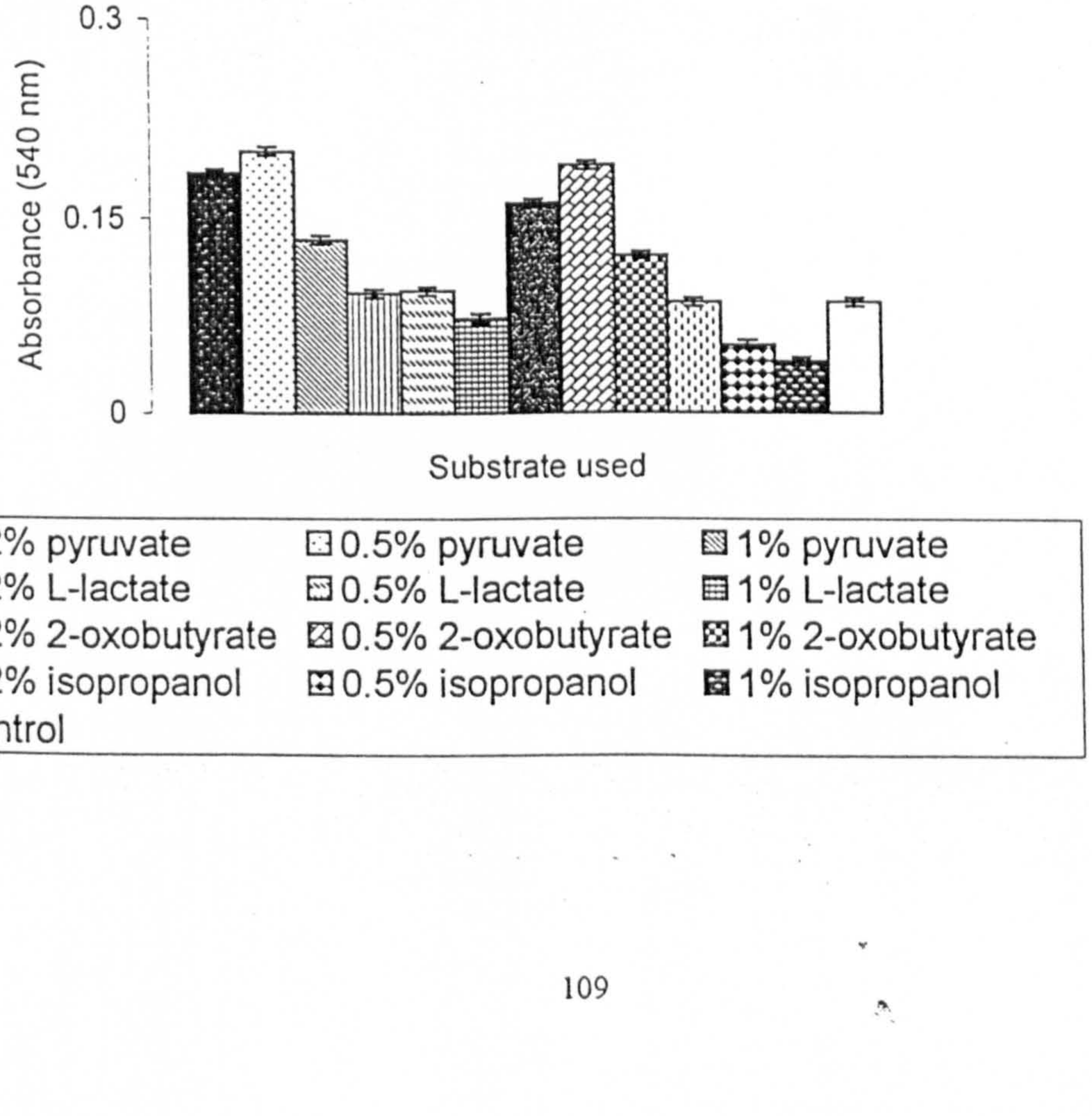


Figure 3.16 The effect of organic acids and isopropanol on the growth of *M. bovigenitalium* strain 398/87. Growth was measured as increase in OD (540 nm) and determined at 24, 48 and 72 hours. Data shown are maximum values, which in all cases were reached at either 24 or 48 hours. Data are means ± SD of three independent experiments.

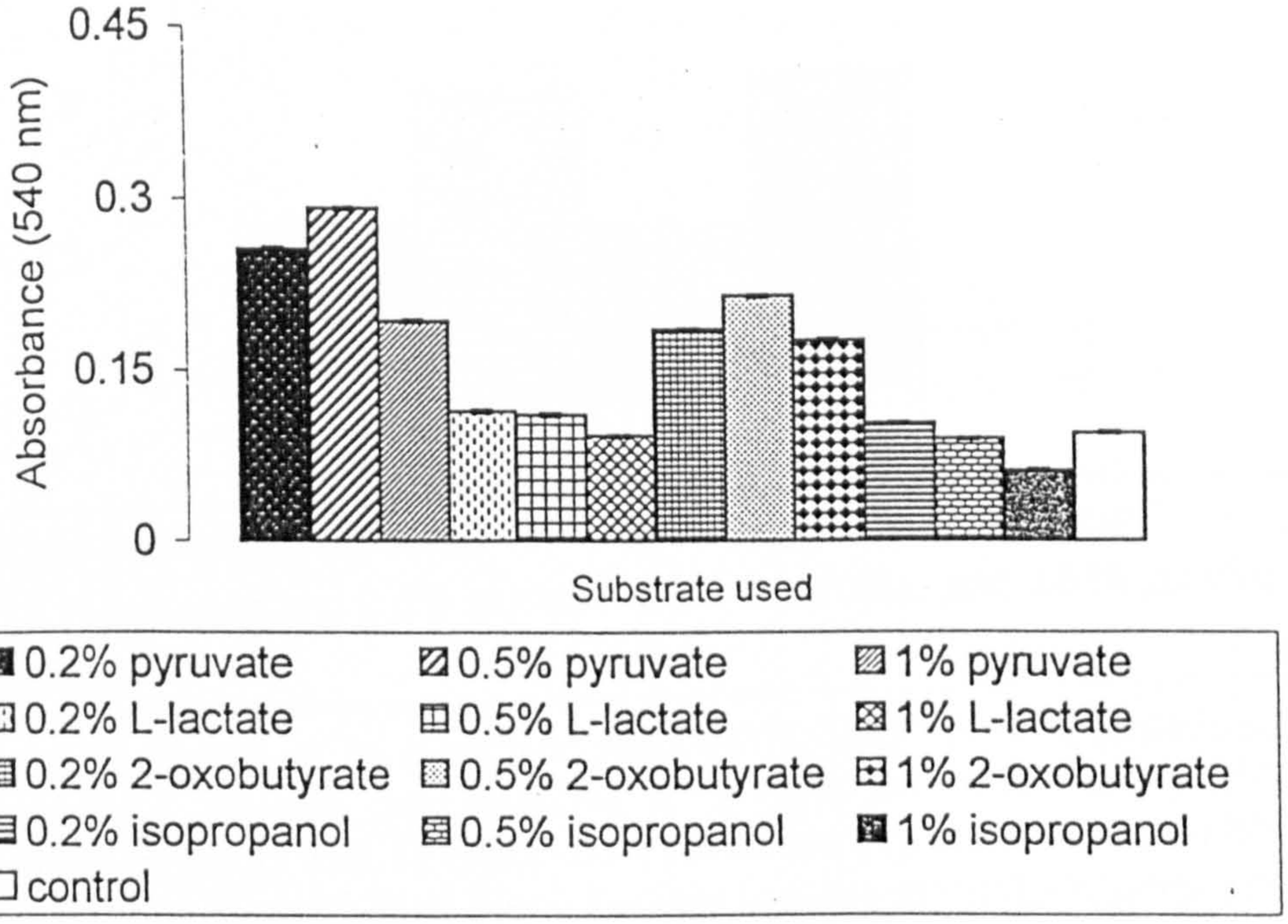


Figure 3.17 Growth of *M. bovis* type strain NCTC 10131 in SP4 medium supplemented with pyruvate. Growth was measured as increased in optical density (540 nm) and determined at 24, 48 and 72 hours. Data shown are maximum values, which in all cases were reached at either 24 or 48 hours. Data are means ± SD of three independent experiments.

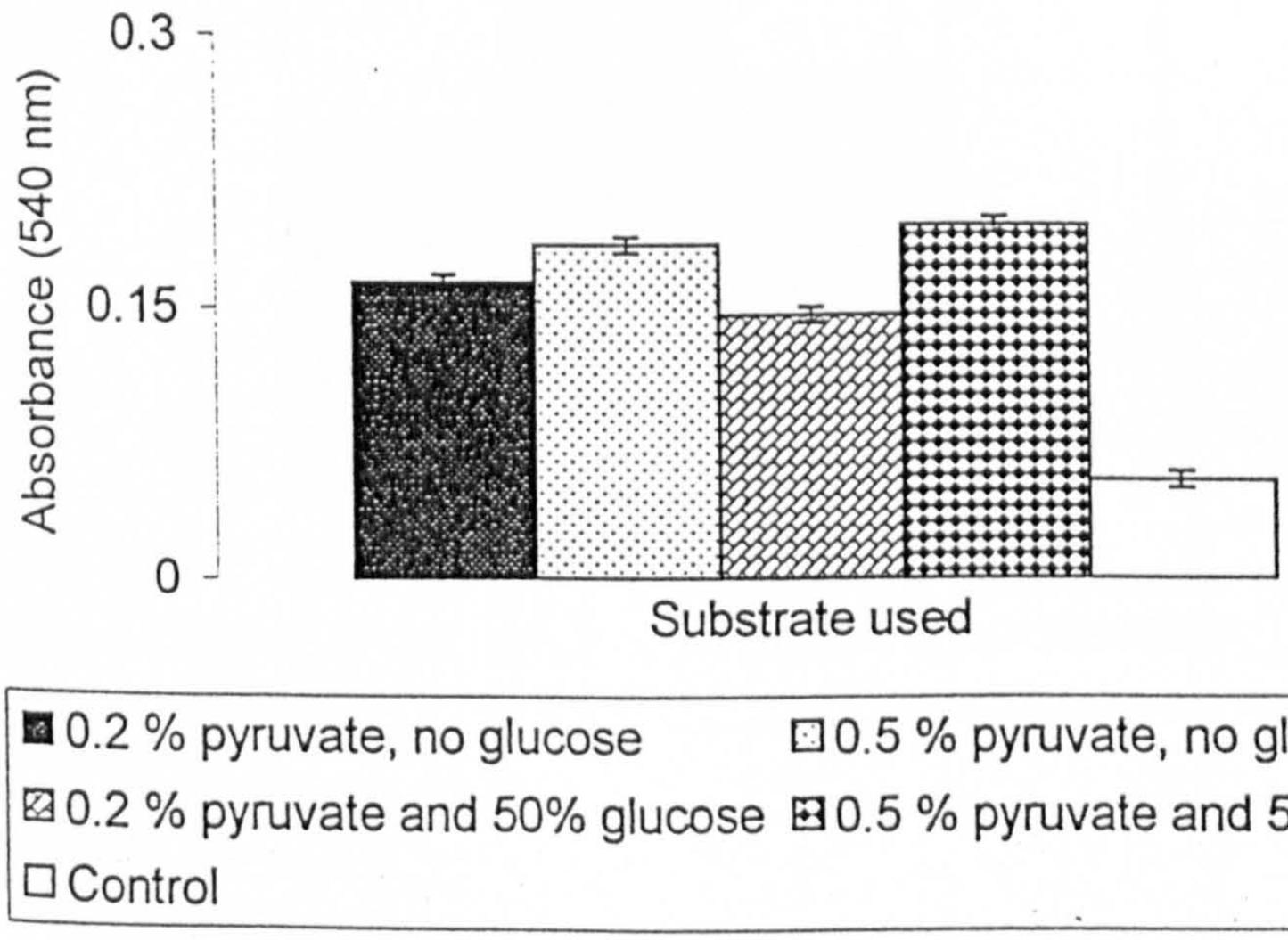


Figure 3.18 Growth of *M. bovis* type strain NCTC 10131 in PP medium supplemented with pyruvate. Growth was measured as increased in optical density (540 nm) and determined at 24, 48 and 72 hours. Data shown are maximum values, which in all cases were reached at either 24 or 48 hours. Data are means ± SD of three independent experiments.

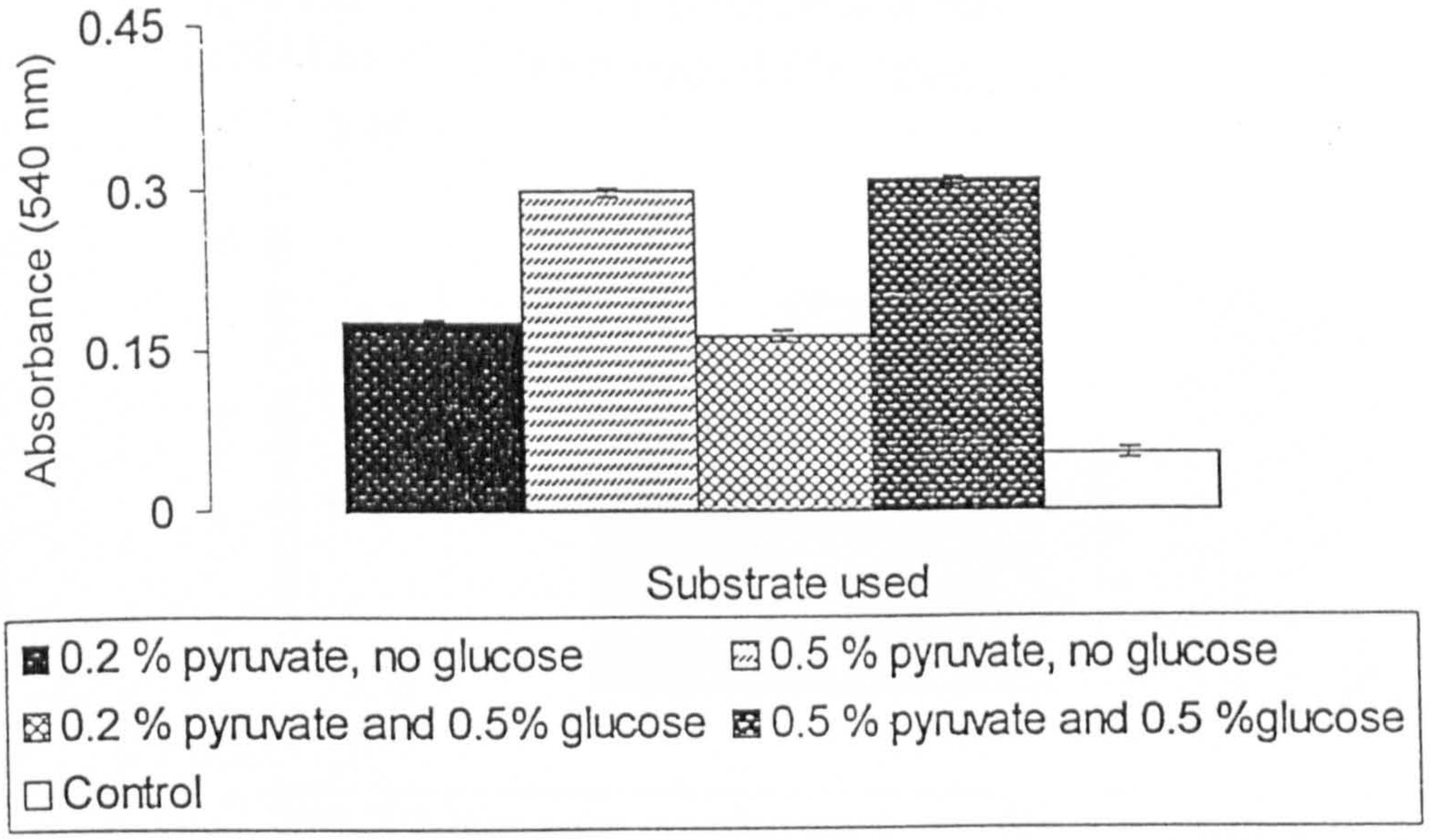


Figure 3.19 Growth of *M. bovis* type strain NCTC 10131 in PRM medium supplemented with pyruvate. Growth was measured as increase in optical density (540 nm) and determined at 24, 48 and 72 hours. Data shown are maximum values, which in all cases were reached at either 24 or 48 hours. Data are means ± SD of three independent experiments.

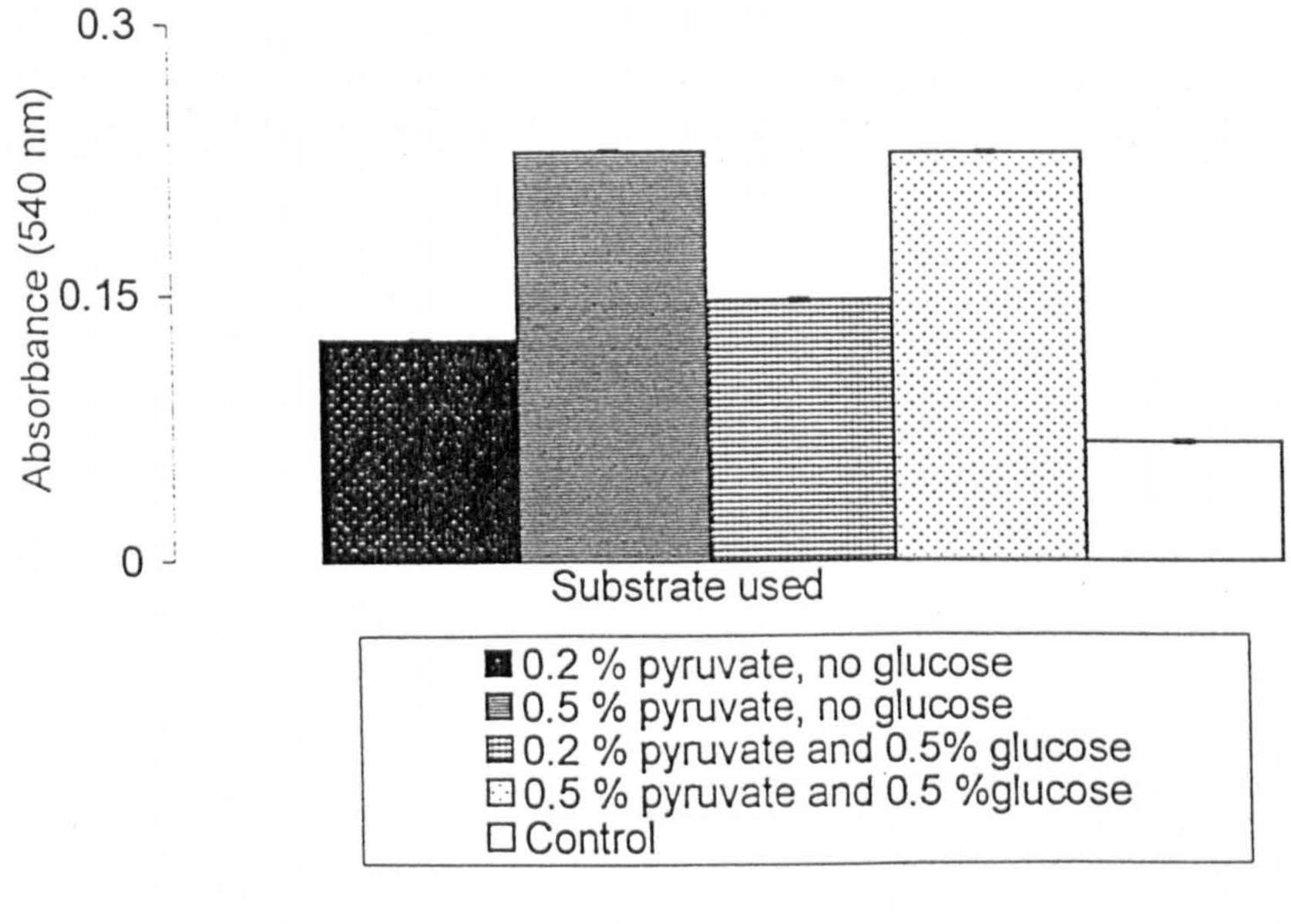


Figure 3.20 Growth of *M. bovis* type strain NCTC 10131 in Eaton's medium supplemented with pyruvate. Growth was measured as increase in optical density (540 nm) and determined at 24, 48 and 72 hours. Data shown are maximum values, which in all cases were reached at either 24 or 48 hours. Data are means ± SD of three independent experiments.

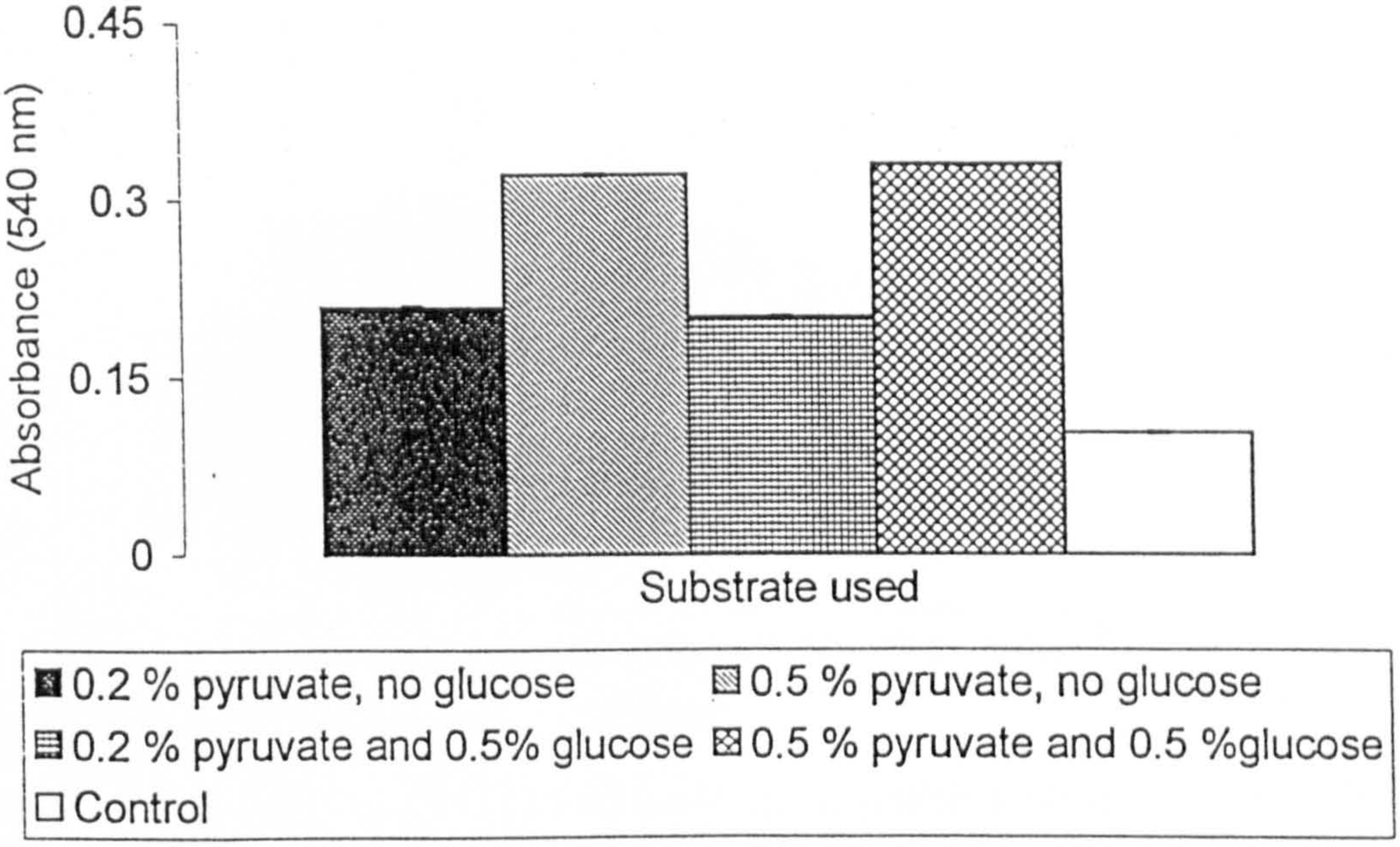


Figure 3.21 Growth of *M. bovis* strain 82B96 in Eaton's medium supplemented with increasing concentration of pyruvate. Growth was measured as increase in OD (540 nm) and determined at 24, 48 and 72 hours. Data shown are maximum values, which in all cases were reached at either 24 or 48 hours. Data are means ± SD of three independent experiments.

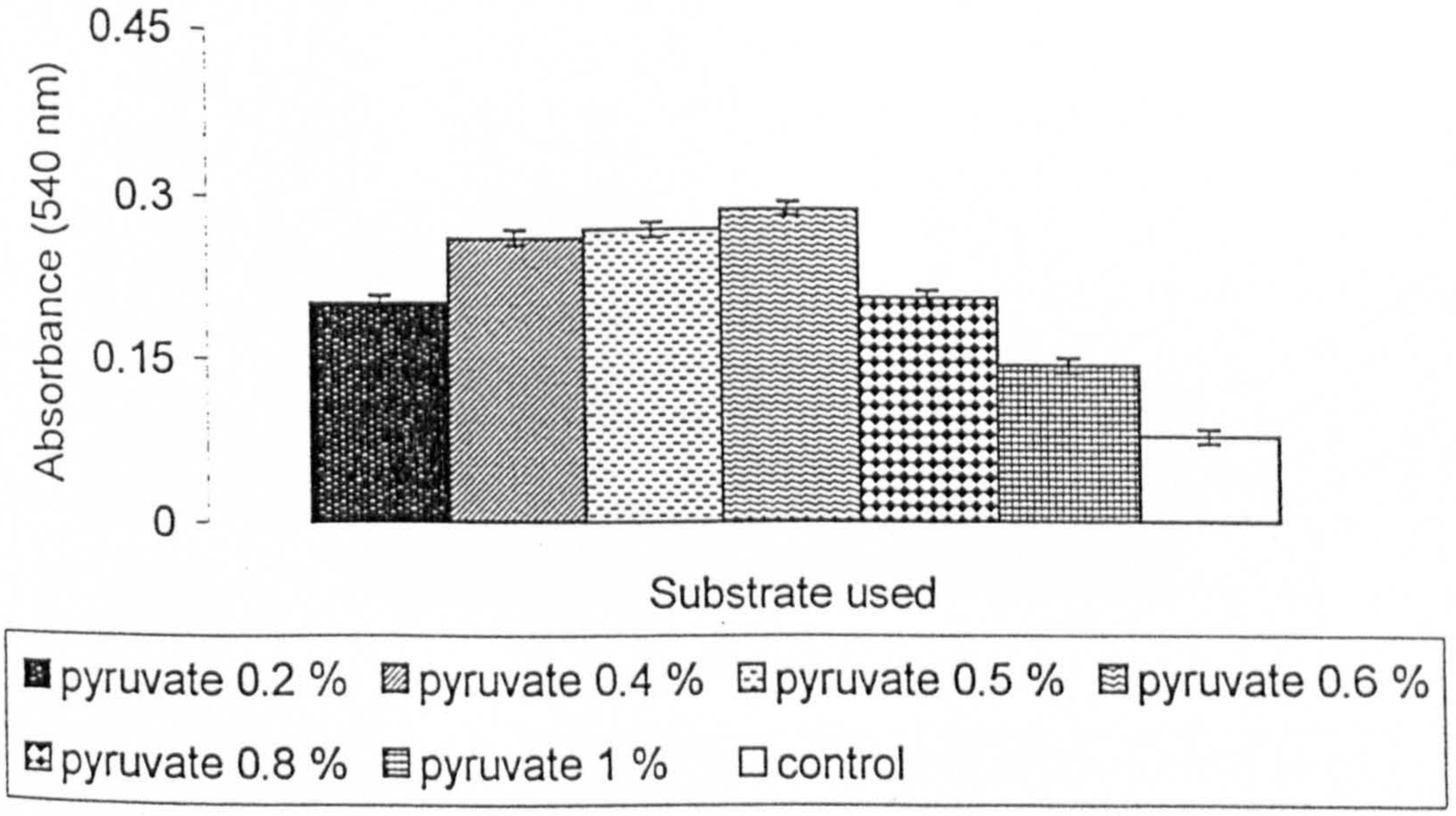


Figure 3.22 Growth of *M. agalactiae* strain 101/94 in Eaton's medium supplemented with pyruvate. Growth was measured as increase in OD (540 nm) and determined at 24, 48 and 72 hours. Data shown are maximum values, which in all cases were reached at either 24 or 48 hours. Data are means ± SD of three independent experiments.

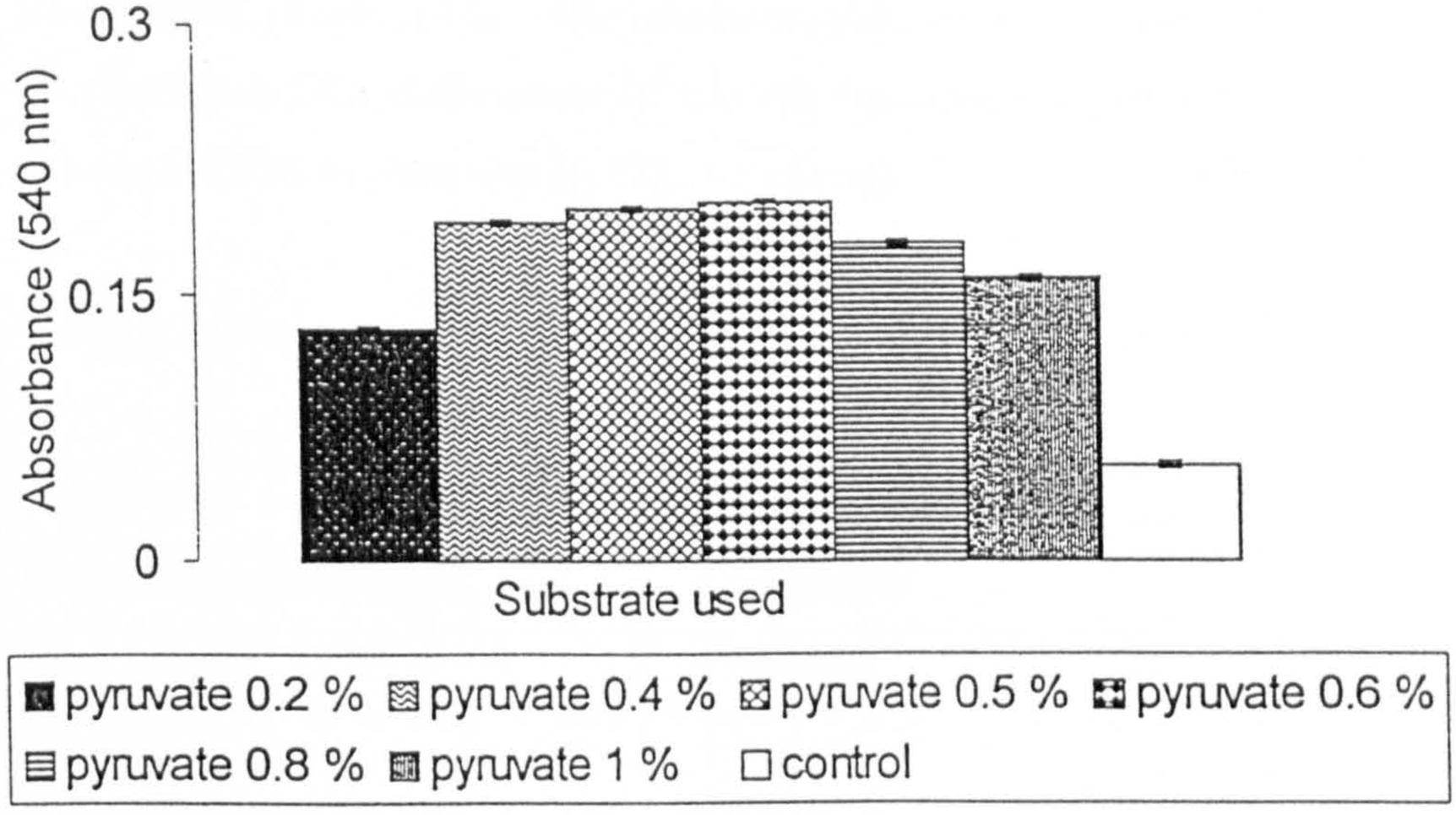
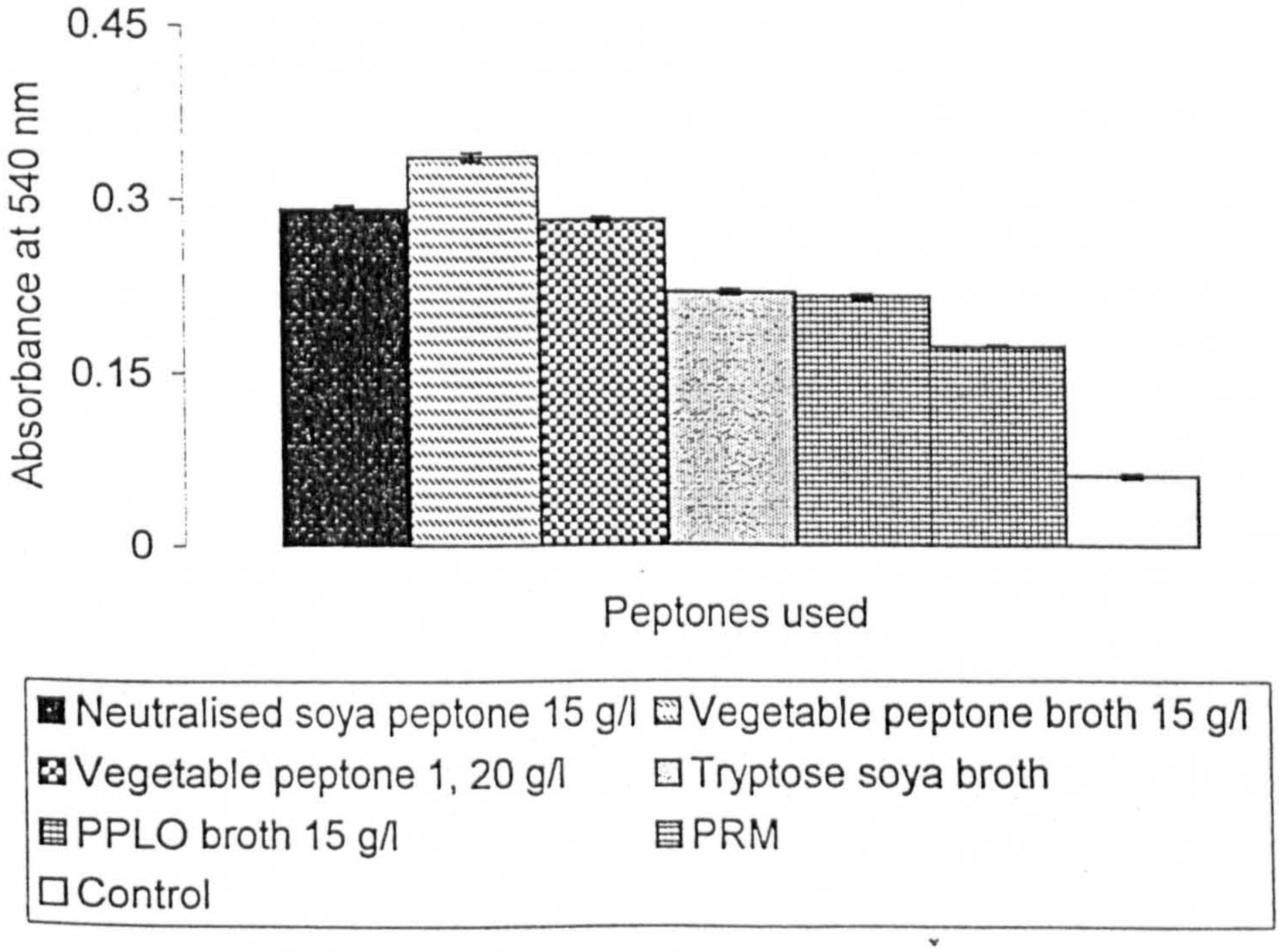


Figure 3.23 The effect of peptones (vegetable and non-vegetable source) on the growth of *M. bovis* 86B96. Growth was measured as increase in optical density (540 nm) and determined at 24, 48 and 72 hours. Data shown are maximum values, which in all cases were reached at either 24 or 48 hours.
Data are means ± SD of three independent experiments.



PRM is a complex medium and was modified by using a vegetable source of peptones (vegetable peptone broth 15 g/l) highest concentration (0.5 % w/v) of pyruvate and glucose was omitted (Table 3.14). The maximum yield of *M. bovis* 86B96 strain in this medium (0.336 culture OD; viable count 10^9 cfu/ml) was obtained. The culture OD and viable count in control PRM medium was (0.173, 10^7 cfu/ml).

Table 3.14 Components of the modified PRM medium

Component	**Quantity per litre**
Vegetable peptone broth	15 grams
Yeast extract	5 grams
Glycerol	5 grams
Sodium chloride	5 grams
Sodium pyruvate	5 grams
HEPES	9 grams
Fresh yeast extract	100 ml
Porcine serum	100 ml

3.5 The effect of inhibitors on the growth of *M. bovis* and *M.* ovine serogroup 11

Effect of different inhibitors on the growth of *M. bovis and M.* ovine serogroup 11 was also determined and maximum concentration of the test inhibitors tolerated by these species was determined. *M. bovis* and *M.* ovine serogroup 11 strains unable to ferment sugars or hydrolyse arginine were grown in the basal broth medium (PRM). Growth of arginine-hydrolysing mycoplasmas is selectively inhibited by L-citrulline, L-lysine and L-ornithine (Ozcan, 1997). L-lysine may compete with arginine for uptake by mycoplasma cells. Citrulline is an intermediate of the arginine hydrolysis pathway and ornithine is an end product, whose transport from cells may be linked to arginine uptake. Fermentative organisms may be inhibited by the addition of fluoride and iodoacetate.

Iodoacetae inhibits the oxidation of glyceraldehyde-3-phosphate to 1,3-diphosphoglycerate and fluoride inhibits enolase, which metabolises 2-phosphoglycerate to

phosphoenolpyruvate. Glucose analogues such as α-methylglucoside are transported into cells and phosphorylated via the phosphoenolpyruvate phosphotransferase system (PEP:PTS) but are not further metabolised. The aim of this study was to determine the maximum tolerable concentrations of these inhibitors by these species. The inhibitors used in the study were α-methylglucoside, citrulline, lysine, ornithine and penicillin.

The growth of *M. bovis* and *M.* ovine serogroup 11 was inhibited at 150 mM for α-methylglucoside, citrulline, lysine, and ornithine. The tolerable concentration of inhibitors for these two species was 100 mM (Figures 3.24, 3.25, 3.26, 3.27, 3.28 and 3.29). The maximum tolerable concentration for penicillin for *M. bovis* was 0.5-1.0 % (w/v). Growth was inhibited at 1.5 % (w/v) (Figure 3.30). The maximum concentration of penicillin for *M.* ovine serogroup 11 strain 2D and 95SR99 was 0.5% (w/v) but the growth of these organisms was inhibited at the concentration of 1-1.5 % (w/v) (Figure 3.31 and 3.32).

Glucose analogue α-methylglucoside is non-metabolisable, taken up by glucose-fermenting organisms and phosphorylated to α-methyl glucoside-6-phosphate by PEP:PTS. *M. bovis* and *M.* ovine serogroup 11 are non-fermentative and it was expected that these species would tolerate a maximum concentrations of this glucose analogue but it was observed that the maximum tolerable concentration was 100 mM. It was also expected that the amino acid citrulline, lysine and ornithine would not cause any adverse effects on the growth of *M. bovis* and *M.* ovine serogroup 11 because these organisms are non-arginine hydrolysing but it was found that the maximum tolerable concentration of these amino acids by these two species was 100 mM. The growth of these organisms can be improved and made selective by the addition of pyruvate $5gl^{-1}$ and inhibitors (α-methylglucoside L-citrulline, L-lysine, L-ornithine and penicillin) of non-target species.

3.6 Conclusions

Substrate oxidation did not show any consistent differences among *M. agalactiae*, *M. bovis*, *M. bovigenitalium* and *M.* ovine serogroup 11. It appears that strains assigned to these groups are adapted to the utilisation of a small range of organic acids and alcohols.

Figure 3.24 Effect of the inhibitor α-methyl glucoside on the growth of *M. bovis* strain 79B96. Growth was measured as increase in OD (540 nm) and determined at 24, 48 and 72 hours. Data shown are maximum values, which in all cases were reached at either 24 or 48 hours. Data are means ± SD of three independent experiments.

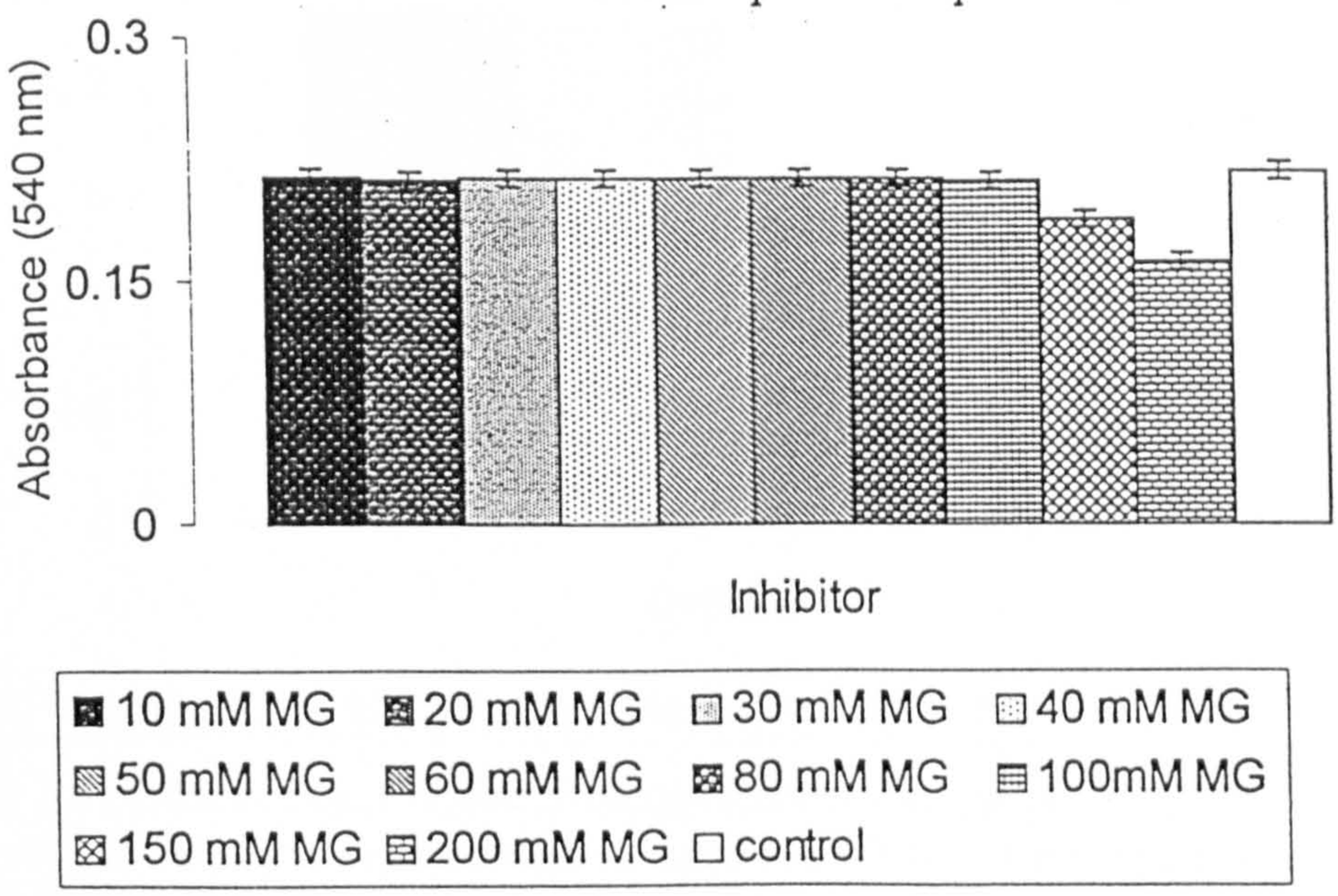


Figure 3.25 Effect of the inhibitors citrulline, lysine and ornithine on the growth of *M. bovis* strain 79B96. Growth was measured as increase in OD (540 nm) and determined at 24, 48 and 72 hours. Data shown are maximum values, which in all cases were reached at either 24 or 48 hours. Data are means ± SD of three independent experiments.

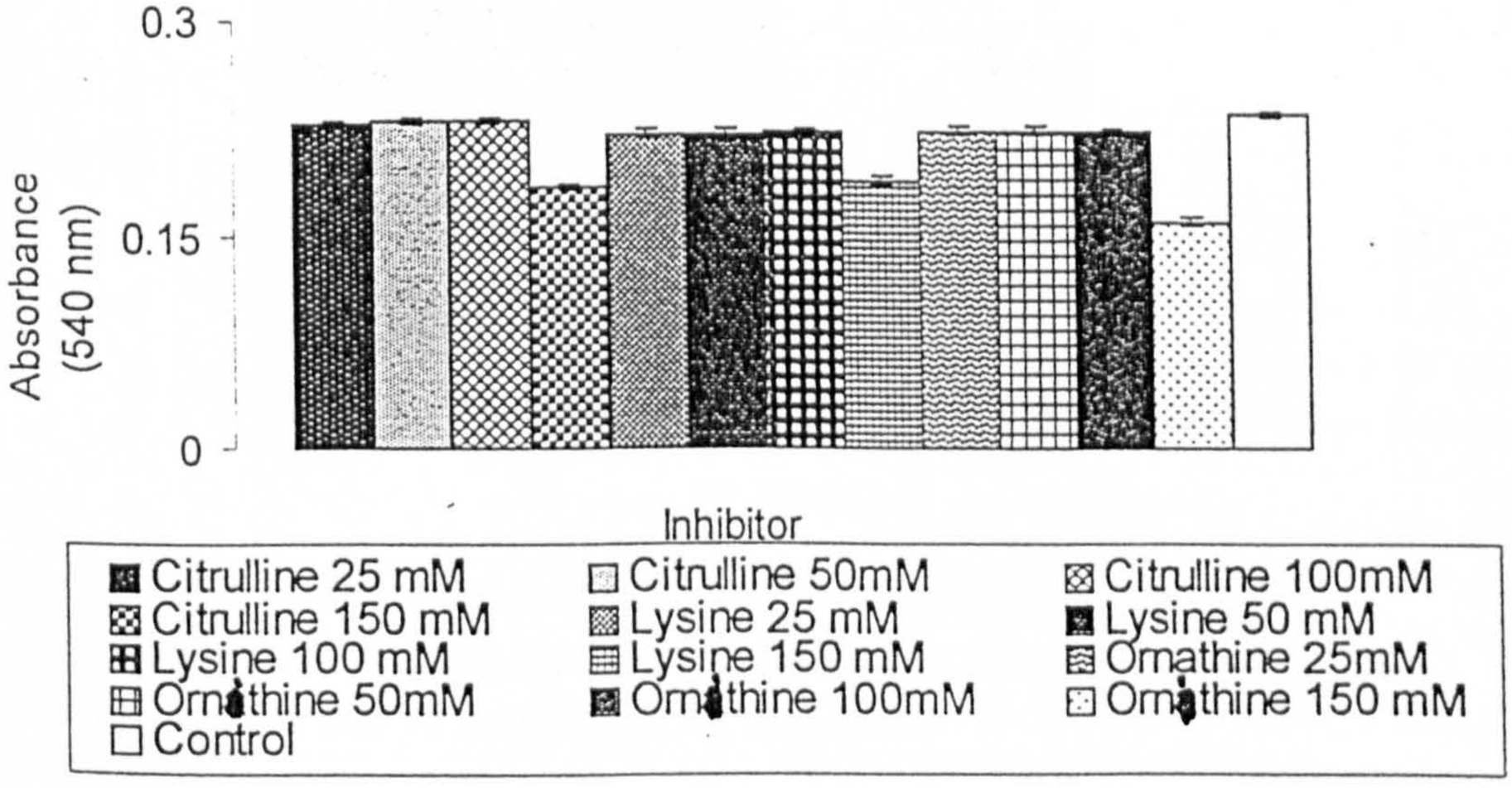


Figure 3.26 Effect of the inhibitor penicillin on the growth of *M. bovis* strain 79B96. Growth was measured as increase in OD (540 nm) and determined at 24, 48 and 72 hours. Data shown are maximum values, which in all cases were reached at either 24 or 48 hours. Data are means ± SD of three independent experiments.

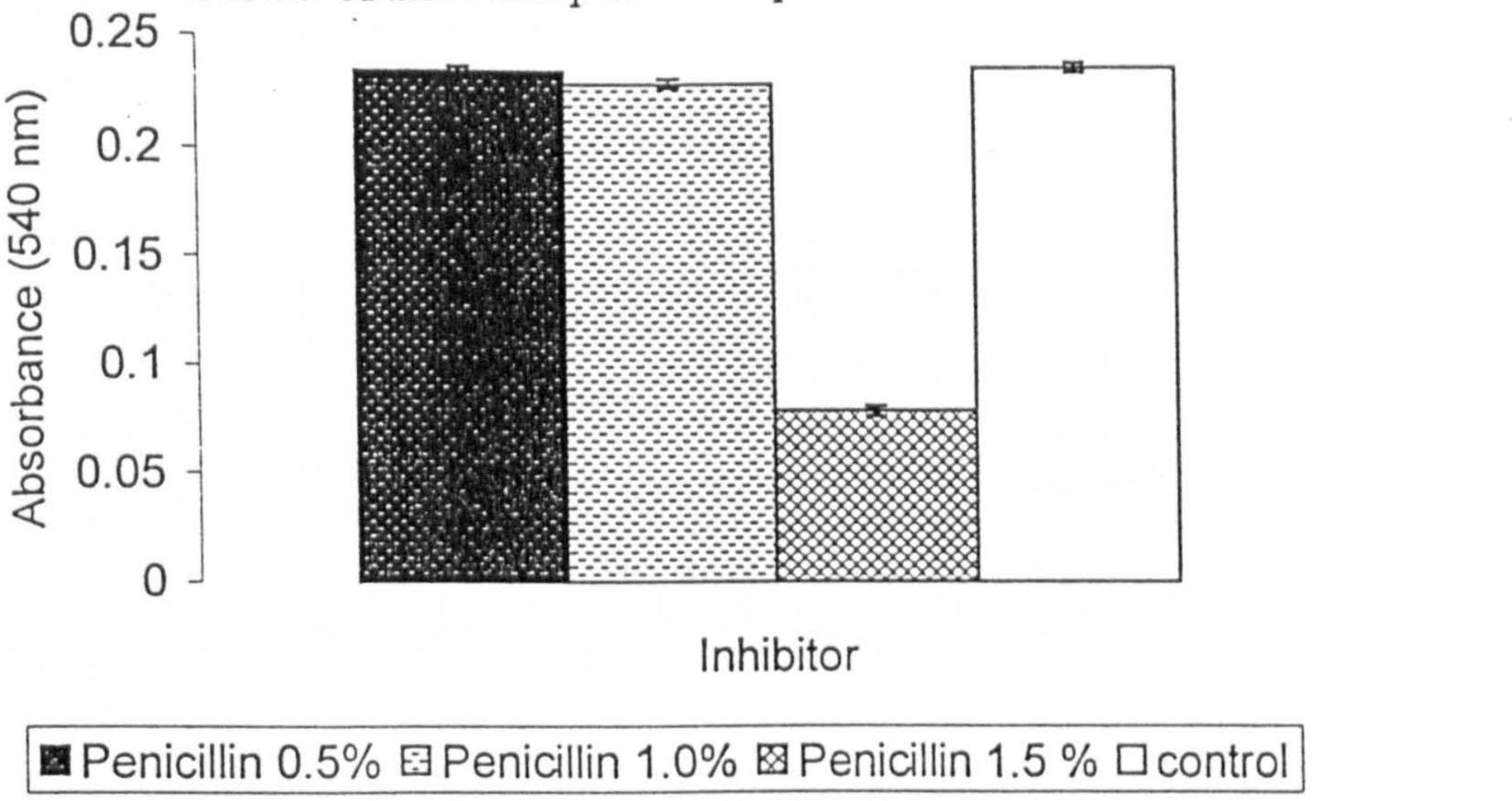


Figure 3.27 Effect of the inhibitor α-methyl glucoside on the growth of *M.* ovine serogroup 11 strain 2D. Growth was measured as increase in OD (540 nm) and determined at 24, 48 and 72 hours. Data shown are maximum values, which in all cases were reached at either 24 or 48 hours. Data are means ± SD of three independent experiments.

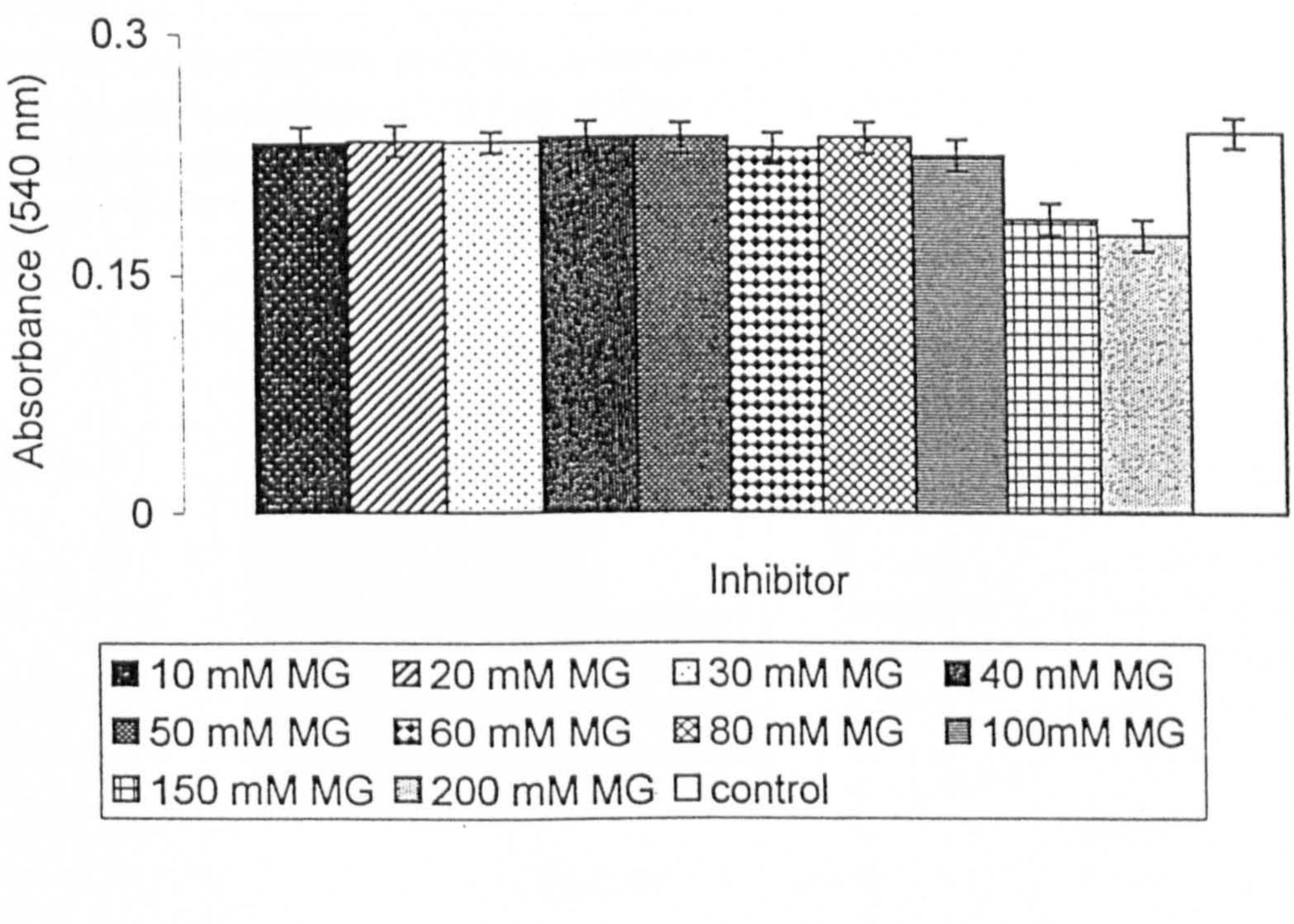


Figure 3.28 Effect of the inhibitors citrulline, lysine and ornithine on the growth of *M.* ovine serogroup 11 strain 2D. Growth was measure as increased in OD (540 nm) and determined at 24, 48 and 72 hours. Data shown are maximum values, which in all cases were reached at either 24 or 48 hours. Data are means ± SD of three independent experiments.

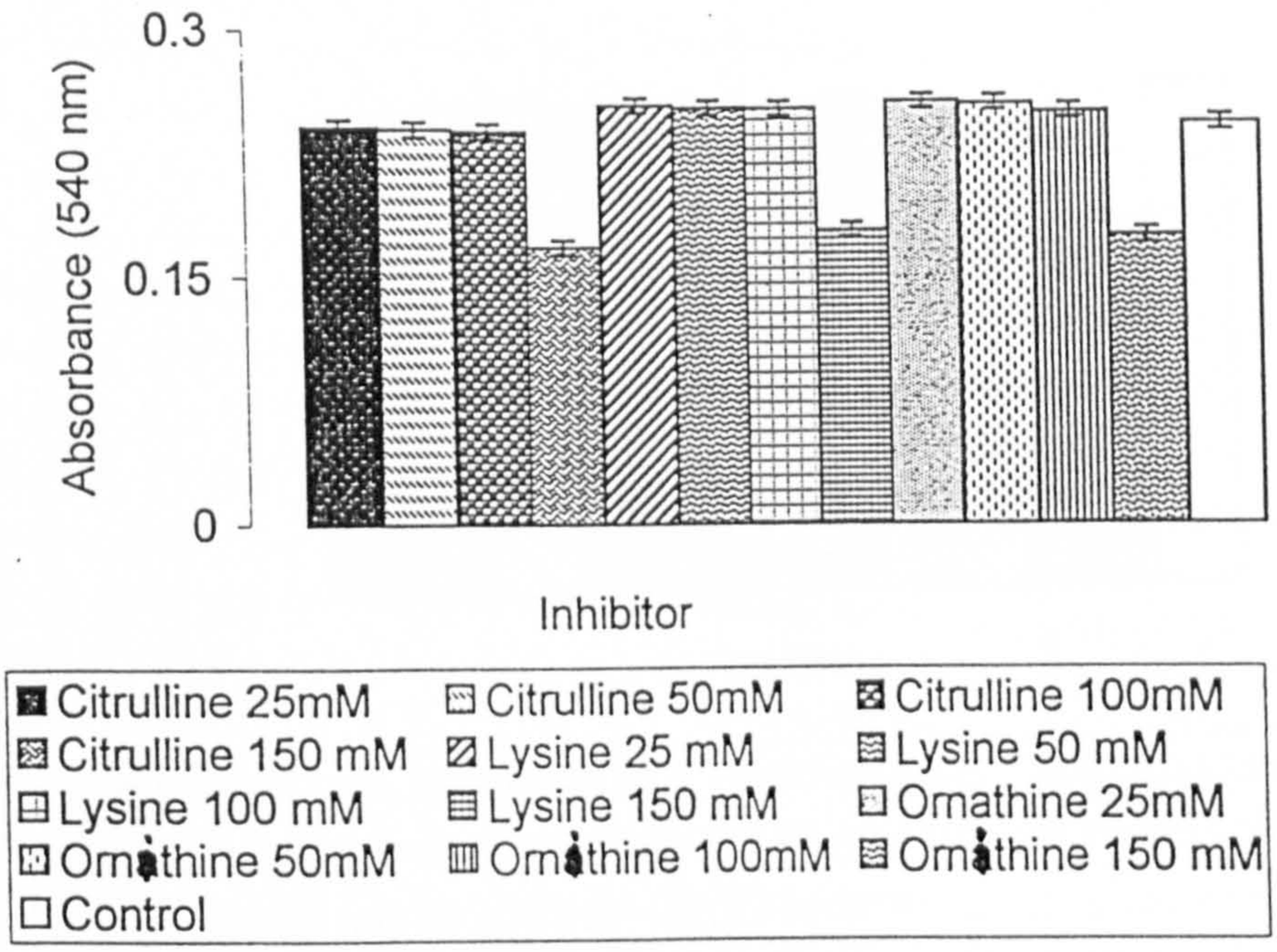


Figure 3.29 Effect of the inhibitor penicillin on the growth of *M.* ovine serogroup 11 strain 2D. Growth was measured as increase in OD (540 nm) and determined at 24, 48 and 72 hours. Data shown are maximum values, which in all cases were reached at either 24 or 48 hours. Data are means ± SD of three independent experiments.

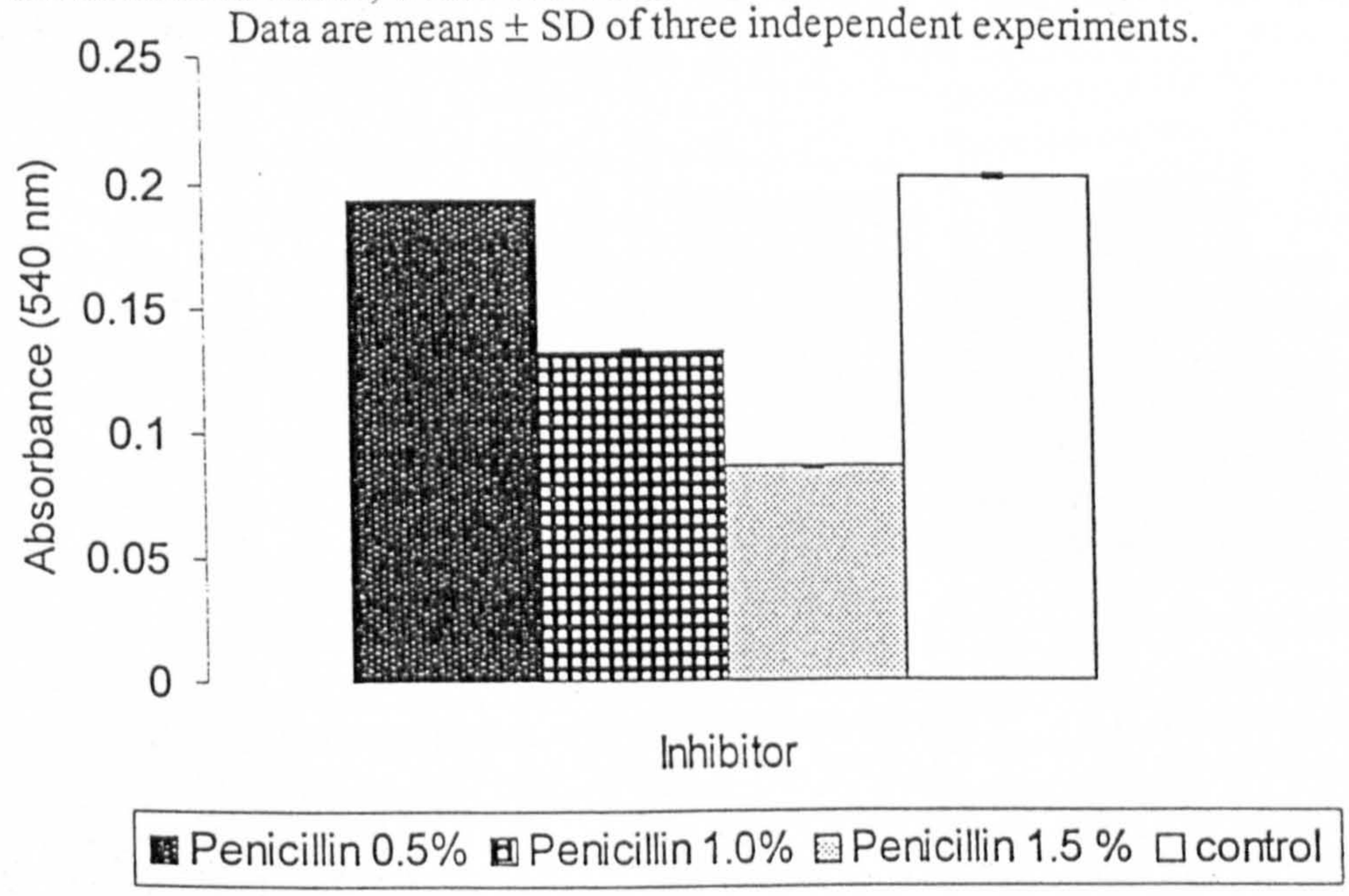


Figure 3.30 Effect of the inhibitor α-methyl glucoside on the growth of *M.* ovine serogroup 11 strain 95SR99. Growth was measured as increase in OD (540 nm) and determined at 24, 48 and 72 hours. Data shown are maximum values, which in all cases were reached at either 24 or 48 hours. Data are means ± SD of three independent experiments.

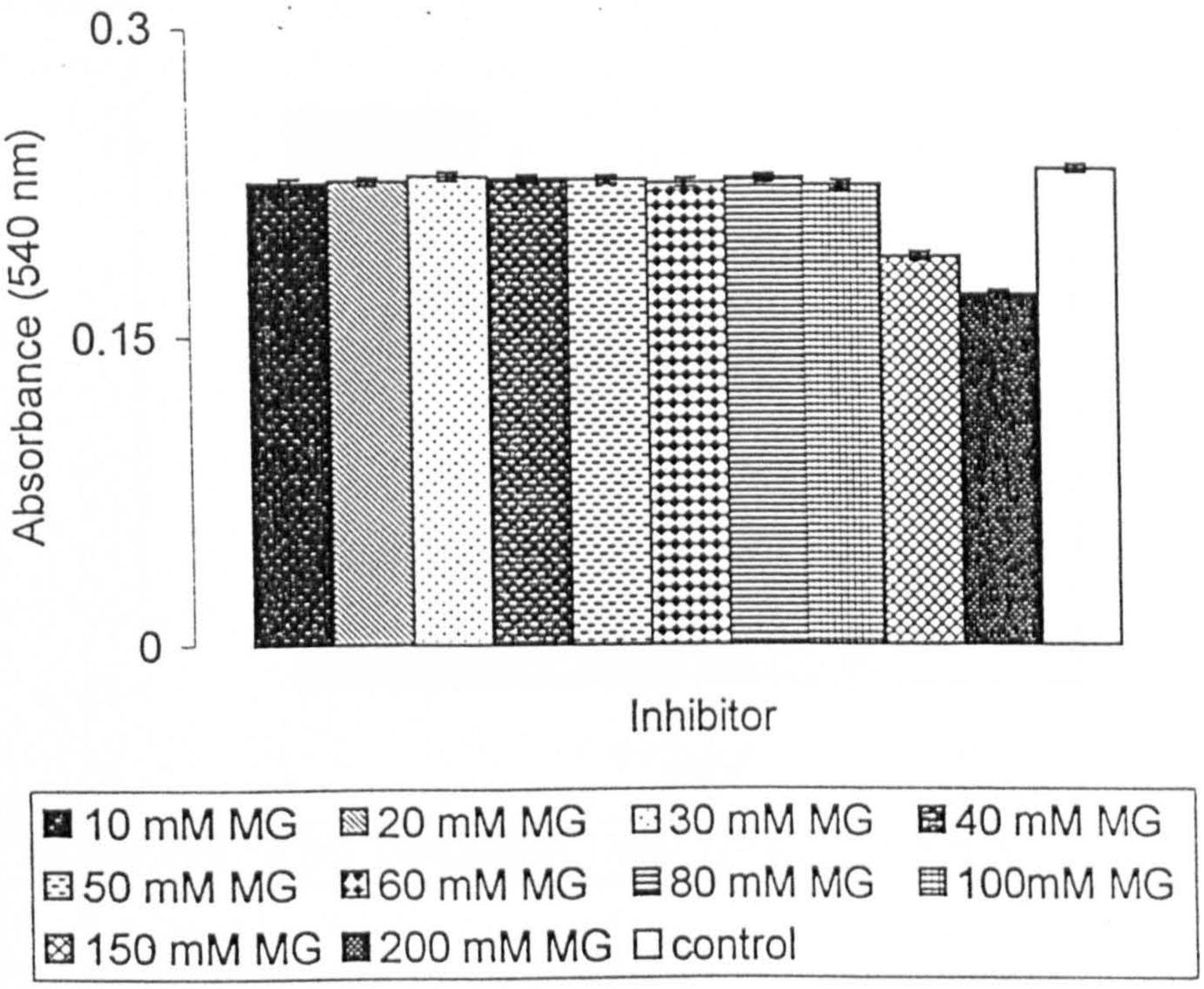


Figure 3.31 Effect of the inhibitors citrulline, lysine and ornithine on the growth of *M.* ovine serogroup 11 strain 95SR99. Growth was measured as increased in OD (540 nm) and determined at 24, 48 and 72 hours. Data shown are maximum values, which in all cases were reached at either 24 or 48 hours. Data are means ± SD of three independent experiments.

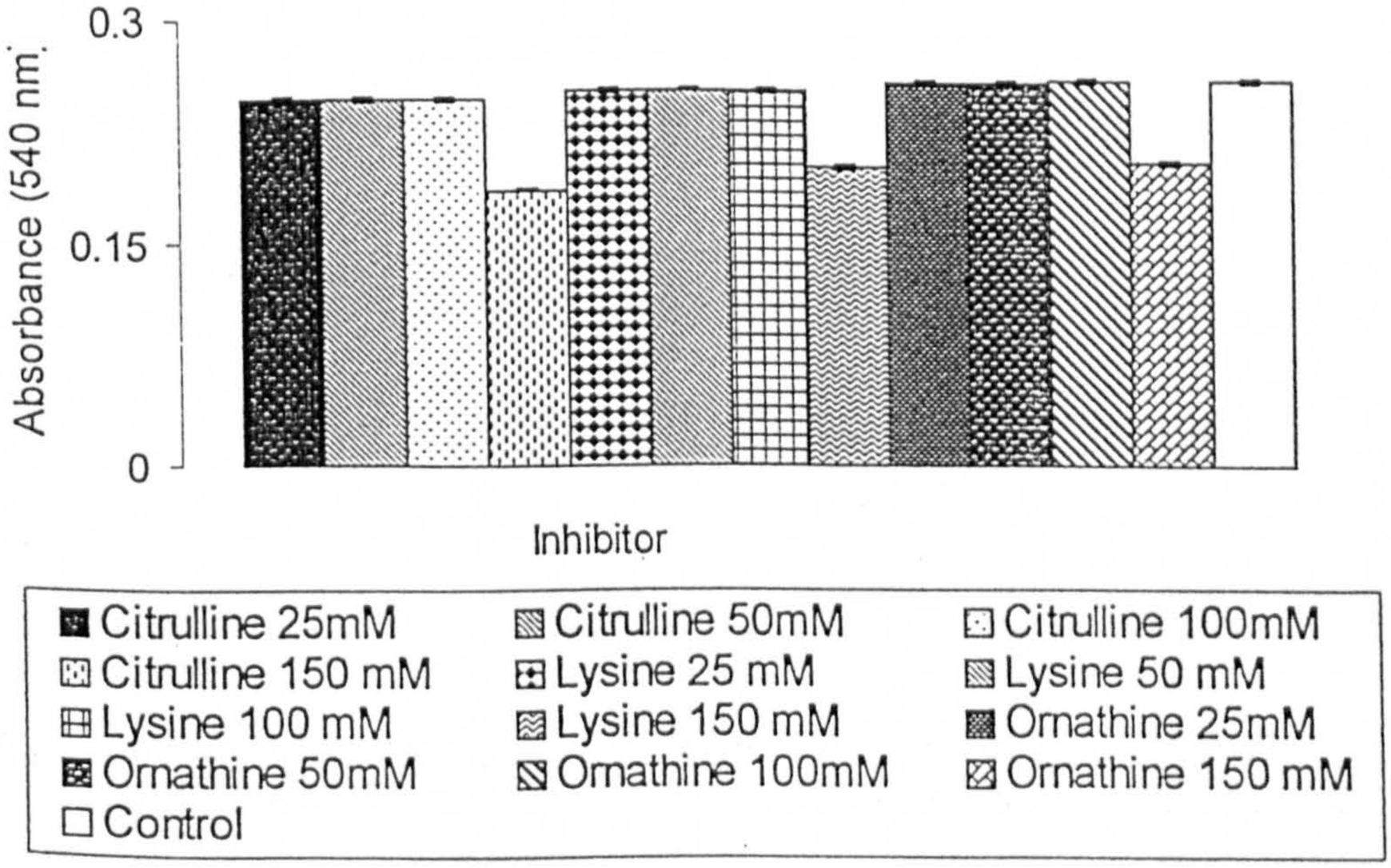
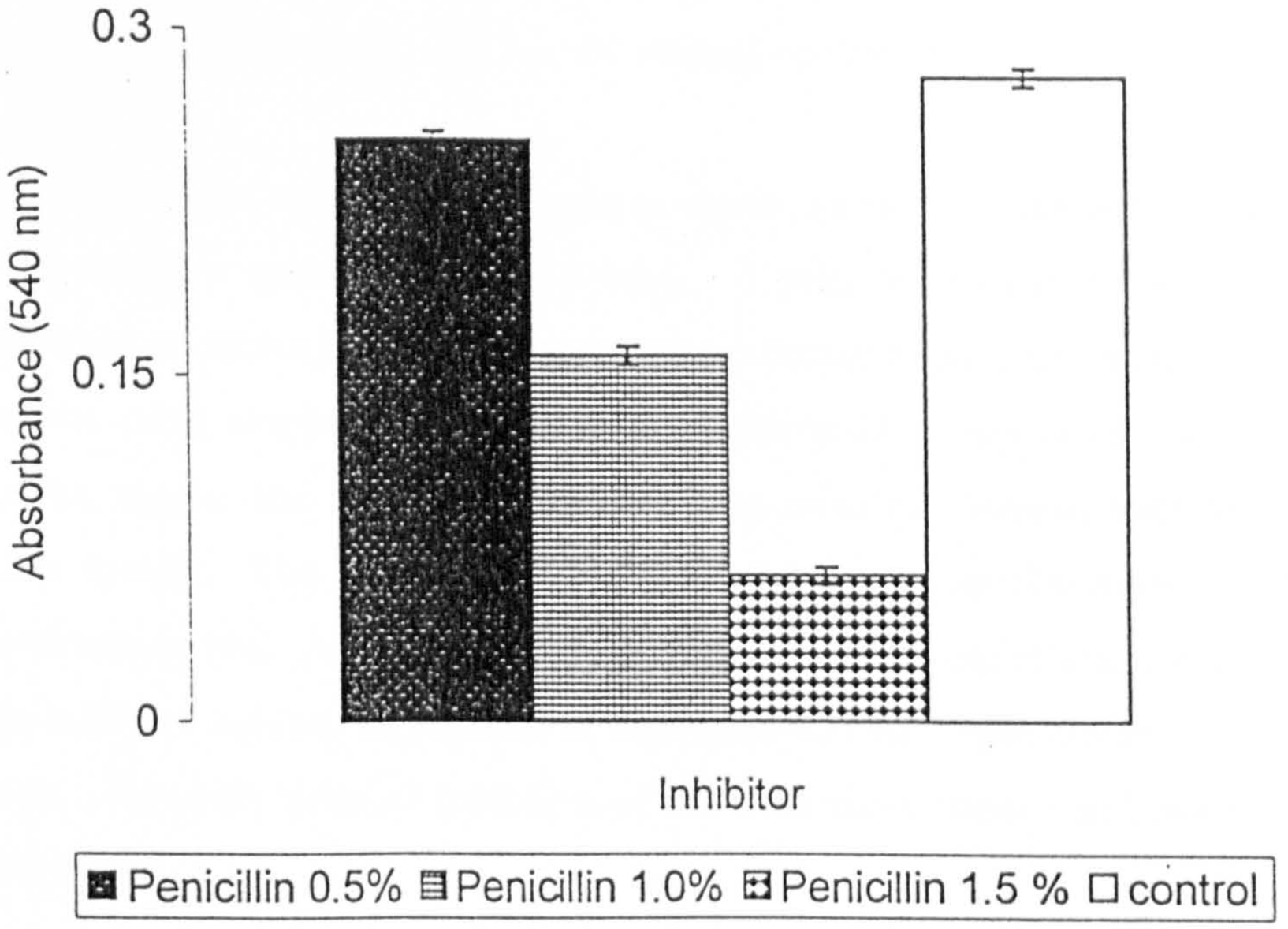


Figure 3.32 Effect of the inhibitor penicillin on the growth of *M.* ovine serogroup 11 strain 95SR99. Growth was measured as increase in OD (540 nm) and determined at 24, 48 and 72 hours. Data shown are maximum values, which in all cases were reached at either 24 or 48 hours. Data are means ± SD of three independent experiments.



The affinity for the substrates metabolised was generally high for L-lactate, 2-oxobutyrate, pyruvate and isopropanol, but low for ethanol and propanol.

All these strains were unable to oxidise sugars, citric acid cycle intermediates and glycerol even at higher concentrations (2.5 mM). A principal reason for determining patterns of substrate oxidation in these species is their potential application in the identification of new isolates using simple and sensitive oxygen electrode system or alternate methods. These results suggest that it is possible to develop rapid biochemical tests for identification of these species. The oxidation of isopropanol by these species might be very useful as a confirmatory test. A consistent feature of all the species and strains in the present study was the ability to oxidise isopropanol at high rates and with high affinity compared to organic acids. However rates of oxidation of other alcohols(ethanol and propanol) tested were usually low.

All SC strains tested were characterised by: an inability to oxidise maltose and trehalose; a low affinity towards glucosamine and mannose; a high affinity towards fructose and N-acetylglucosamine; and an ability to oxidise organic acids (L-lactate, pyruvate and 2-oxobutyrate). African SC strain SH9 appears to be different from European SC strains, as it was able to metabolise glucose at highest concentration with low affinity. European SC strains were also different from African SH9 by their inability to oxidise glycerol. The patterns and kinetics of substrate oxidation appeared to be a generally stable feature amongst non-fermentative and non-arginine hydrolysing mycoplasmas and SC strains. The results presented in this chapter suggest that patterns of substrate oxidation might be useful in the preliminary screening of *Mycoplasma* isolates from ruminants prior to serological tests. The data derived here were based on the determination of oxygen uptake, measured by changes in DOT of washed cell suspensions. Following this method it may be possible to test for the presence of enzymes responsible for the degradation of specific substrates. Wadher and Miles (1988) showed that α-glucosidase which is responsible for maltose utilisation was present in the type strain of *M. mycoides* subsp. *capri*.

Chapter 4

4. Hydrogen peroxide production by members of *Mycoplasma agalactiae, M. bovis, M. bovigenitalium, M.* ovine serogroup 11 and *M. mycoides* SC

4.1 Introduction

The appearance of oxygen on earth led to two major problems: the production of potentially deleterious reactive oxygen species and a drastic decrease in iron availability. Fridovich (1995) described reactive oxygen species (ROS) as deleterious in excess. They are naturally produced by aerobic metabolism and are a permanent threat to living organisms. The generation of ROS such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), hypochlorous acid (HCIO), and hydroxyl radical (OH^-) is the most extensively studied mechanism of micro-organism killing by vertebrate phagocytes (Rosen *et al.*, 1995). O_2^- is produced by the NADPH oxidase complex found in phagocyte membranes. This enzymatic complex catalyses the reduction of molecular oxygen to O_2^- using NADPH as electron donor. At physiological pH, O_2^- quickly dismutases to H_2O_2, which is converted to OH^- in the presence of metal ions (e.g. Fe^{2+}) by the Fenton reaction. In the presence of peroxidase, H_2O_2 oxidises chlorine to HCIO, which at physiological pH produces hypochloride anion, a potent microbicidal agent. The ROS generated during phagocytosis are also toxic for host cells, which use superoxide dismutase (SOD) and catalase (CAT) to remove O_2^- and H_2O_2 respectively.

McLeod and Gordon (1923) first reported the production of H_2O_2 by pneumococcal bacteria, and others have demonstrated that peroxide is generated by many micro-organisms (Dahiya and Speck, 1968; Kekessy and Pignet, 1970). H_2O_2 production has been demonstrated in a wide range of mycoplasmas including *M. arthritidis, M. bovigenitalium, M. canis, M. dispar, M. gallinaceum, M. pneumoniae, M. pullorum, M. pulmonis* and members of the *M. mycoides* cluster (Binder *et al.*, 1990; Miles *et al.*, 1991; Abu-Groun, 1992; Taylor *et al.*, 1996).

The production and release of H_2O_2, superoxide ions and possibly other reactive species (OH^-) are believed to play an important role in mycoplasma pathogenicity. H_2O_2 is an oxidising agent and also reacts with iron and copper ions in cells and body fluids to form free radicals including superoxide and the highly toxic hydroxyl radicals

(Halliwell and Gutteridge, 1995). Miles *et al.* (1991) reported H_2O_2 may be formed during the oxidation of sugars, organic acids or glycerol. In those species able to oxidise substrates to acetate plus CO_2 there is a net production of NADH. *Mycoplasma* species lack quinones and cytochromes and the NADH produced is oxidised by cytoplasmic NADH oxidase (Pollack *et al.*, 1997) with the consumption of molecular oxygen. Active metabolic intermediates such as H_2O_2 (Cole *et al.*, 1968; Niang *et al.*, 1998), galactan (Lloyd *et al.*, 1971) adhesins (Baseman *et al.*, 1982) and variable surface membrane antigens (Wise *et al.*, 1992) have been suggested as potential virulence factors of mycoplasmas. The rapid production and accumulation of active oxygen species (AOS), primarily H_2O_2, have been shown to occur in several plant-microbe interactions and are recognised as the oxidative burst (Lamb and Dixon, 1997). Increased AOS production may exert a toxic effect within cells via lipid peroxidation, protein degradation and DNA damage (Wolff *et al.*, 1986; Imlay and Linn, 1988). Moreover, H_2O_2 production in plants is reported to be a virulence factor (Xiu and Pan, 2000) and it has been hypothesised to act as a signal molecule in the induction of systemic and local acquired resistance (Chen *et al.*, 1993). The antimicrobial effect of exogenously added H_2O_2 has been demonstrated against fungi (Peng and Kuc, 1992) and bacteria (Kiraly *et al.*, 1993).

Two H_2O_2-forming enzymes have been identified in mycoplasmas: NADH oxidase and L-α-glycerophosphate (GP) oxidase. The formation of H_2O_2 by NADH oxidase activity will be dependent upon whether species possess the ability to metabolise sugars and/or organic acids to acetate plus CO_2. This partial oxidation requires the reduction of NAD^+ to NADH. In contrast, where sugars are metabolised only to lactate, there is no net NADH production (Miles, 1992b). In addition, Miles *et al.* (1991) showed that the amount of H_2O_2 produced during NADH oxidation by fermentative *Mycoplasma* species (*M. dispar*, *M. mycoides, M. pneumoniae* and *M. putrefaciens*) varied from 0.05 to 0.20 mol per mol O_2 taken up. Taylor *et al.* (1996) reported a similar variation in the proportion of H_2O_2 formed during NADH oxidation by glucose-and organic acid-oxidising avian mycoplasmas (from 0.02 to 0.32 mol per mol O_2 taken up). This data suggests the presence of two NADH oxidase activities, one yielding H_2O and the other H_2O_2. Furthermore, they suggested the possibility that pathogenicity might be modified by alterations in the ratio of the activities. In *Mycoplasma* species unable to oxidise glucose or organic acids, and which therefore do not produce significant quantities of

NADH, its oxidation led to the production of 1.0 mol H_2O_2 per mol O_2 taken up. Thus, these species may lack H_2O-producing NADH oxidase activity (Miles *et al.*, 1991).

L-α-glycerophosphate oxidase (GPO) is part of the glycerol oxidising pathway in *Mycoplasma* species (Rodwell, 1967; Figure 4.1). The enzyme oxidises GP, formed by the phosphorylation of glycerol in a reaction requiring ATP and catalysed by glycerol kinase. GP oxidation is irreversible and leads to the formation of triose phosphate (dihydroxyacetone phosphate, DHAP), with the production of one mol of H_2O_2 per mol GP oxidised (Miles *et al.*, 1991; Abu-Groun *et al.*, 1994). GPO has been demonstrated in all of the glycerol-oxidising *Mycoplasma* species that have been investigated (including *M. canis, M. dispar* and *M. pneumoniae*) but is not detectable in non-glycerol oxidising strains (Miles *et al.*, 1991; Taylor *et al.*, 1996).

A major aim of the work presented here was to investigate the products of NADH oxidase activity and to quantify H_2O_2 production by newly isolated strains of non-fermentative and non-arginine hydrolysing species of *M. bovis, M. agalactiae, M. bovigenitalium* and *M.* ovine serogroup 11. Differences in production of H_2O_2 amongst mollicutes were studied because of their potential values in the characterisation of subgroups within mollicutes. The possibility was considered that field strains might show variations in H_2O_2 production, showing differences in virulence. In the work conducted therefore, H_2O_2 production during the oxidation of NADH by lysed cells and of pyruvate and other substrates by whole cells, was also investigated. In all experiments, H_2O_2 produced during substrate metabolism was estimated from the increase in DOT of cell suspensions following addition of catalase. It was shown in Chapter 3, that glycerol-oxidising ability was not found amongst the members of *M. agalactiae, M. bovis, M. bovigenitalium and M.* ovine serogroup 11. So another aim of the work presented here was to investigate enzymes specific to the glycerol oxidising pathway.

Figure 4.1 Glycerol oxidation pathway in *M. mycoides*

glycerol +ATP ⟷ L-α-glycerophosphate (GP) [glycerol kinase]

GP + O_2 ⟶ triose phosphate + H_2O_2 [GP oxidase]

triose phosphate ⟶ acetate + CO_2 [several enzymes]

4.2 Results and discussion

4.2.1 NADH oxidation and H_2O_2 production by lysed cells of *M. agalactiae, M. bovis, M. bovigenitalium* and *M.*ovine serogroup 11.

In the experiments conducted here all of the test strains number in parenthesis were non-fermentative and non-arginine hydrolysing mycoplasmas such as *M. agalactiae* (22), *M. bovis* (16), *M. bovigenitalium* (4) and *M.* ovine serogroup 11(12). Washed cells of all the strains were prepared as previously described in Section 2.7 except catalase was omitted and H_2O_2 production was monitored from increase in DOT. All strains of *M. agalactiae* showed variable NADH oxidase activity (36-928 nmol/min/mg cell protein) and the quantities of H_2O_2 produced were also variable (0-1.21 mol per mol oxygen taken up). All *M. agalactiae* strains produced <0.5 mol of H_2O_2 per mol of oxygen taken up except strains 453/94 and 101/94 which produced >1 mol of H_2O_2 per mol of oxygen taken up (Table 4.1). In these two strains H_2O_2 produced was relatively high. H_2O_2 production in *M. agalactiae* type strain and 1070/93 strains was <0.1 mol H_2O_2 per mol of oxygen taken up (Figure 4.2)

All strains of *M. bovis* showed variable NADH oxidase activity (16-329 nmol/min/mg cell protein). Also, the quantities of H_2O_2 produced were variable (0-1.1 of H_2O_2 per mol of oxygen taken up); with some strains produced small quantities (< 0.5 of H_2O_2 per mol of oxygen taken up) and some produced a high quantities (> 0.5 of H_2O_2 per mol of oxygen taken up). Strains of *M. bovis* 119B96 and 136B96 produced approximately 1 mol of H_2O_2 per mol of oxygen taken up (Table 4.2), and these results were thus similar to *M. agalactiae* strains, which also produced approximately 1 mol H_2O_2 per mol O_2 taken up. *M. bovis* type strain 10131 did not produce H_2O_2 and 81B96 strain produced <1 H_2O_2 per mol of oxygen taken up (Figure 4.3). Most strains presumably contained both H_2O and H_2O_2 producing NADH oxidase activities, these strains (Figure 4.4) (119B96 and136B99) might possess only a single H_2O_2 producing activity.

The high yield of H_2O_2 during NADH oxidation by these strains was confirmed several times. These results are in contrast to the results of Abu–Groun *et al.* (1994), where lactate oxidising species of *M. agalactiae* also had a high NADH oxidation activity, which produced little H_2O_2. However the NADH oxidase activity and production of H_2O_2 was shown to be variable in lactate oxidising mycoplasmas studied.

Figure 4.4 H_2O_2 production during oxidation of organic acids by *M. agalactiae*, *M. bovis*, *M. bovigenitalium* and *M.* ovine serogroup 11

Oxidation of sugars and organic acids occurs *via* NAD^+ reduction

$$\text{Substrate} + NAD^+ \longrightarrow \text{acetate} + CO_2 + NADH$$

NADH is then re-oxidised by NADH oxidase:

$$NADH + O_2 \longrightarrow NAD^+ + ?H_2O + ?H_2O_2$$

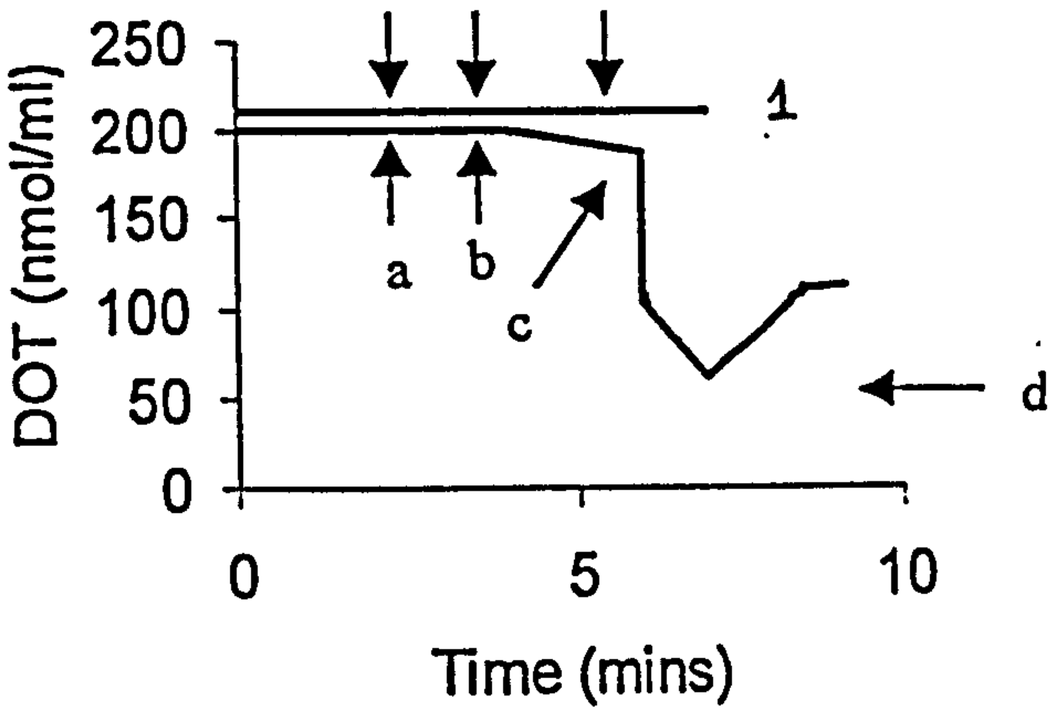
Miles *et al.* (1991) and Taylor *et al.* (1996) reported that all the glucose and organic acid oxidising mycoplasmas that have been studied possess a high NADH oxidase activity, which typically results in the production of only small quantities of H_2O_2 (< 0.1 mol per mol O_2 taken up). However, Abu-Groun (1992) found that of more than fifty strains of the *M. mycoides* cluster studied, three strains gave approximately 1 mol H_2O_2 per mol O_2 taken up.

The NADH oxidase activity in *M. bovigenitalium* and *M.* ovine serogroup 11 was very high (244-1039; 171-1412 nmol/min/mg cell protein respectively) compared to *M. agalactiae* and *M. bovis* but the H_2O_2 production was less in these two species (<0.5 of H_2O_2 per mol of oxygen taken up). All these strains of *M. bovigenitalium* and *M.* ovine serogroup 11 oxidised NADH at relatively high rates and produced little H_2O_2 (Table 4.3). H_2O_2 production by *M.* ovine serogroup 11 2D and 50SR98 was <0.5 of H_2O_2 per mol of oxygen taken up; however *M. bovigenitalium* strain 434/81 and 398/87 produced much less H_2O_2 <0.1 of H_2O_2 per mol oxygen taken up (Figure 4.5 and 4.6).

Taylor *et al.* (1996) have suggested that the species capable of oxidising substrates like glucose or organic acids have two NADH oxidases, with the predominant activity yielding H_2O. However nonoxidative species may have a single NADH oxidase activity. Miles *et al.* (1991) and Taylor *et al.* (1996) showed that *Mycoplasma* species, which oxidised glucose (to acetate), had a relatively high NADH oxidase activity, which characteristically yielded small quantities of H_2O_2 (typically $\leq$ 0.1 mol per mol of O_2 taken up).

Figure 4.2 H_2O_2 production during NADH oxidation by *M. agalactiae* NCTC 10123 (A) and 1070/93 strain (B). Cell suspensions were lysed with Triton-X 100 at point 'a', GP (2.5 mM) was added at point 'b', and NADH (15 µM) was added at point 'c'. Catalase (5µl, 80 mg/ml) was added at point 'd' when oxygen uptake had ceased. Curve 1 is the control.

A



B

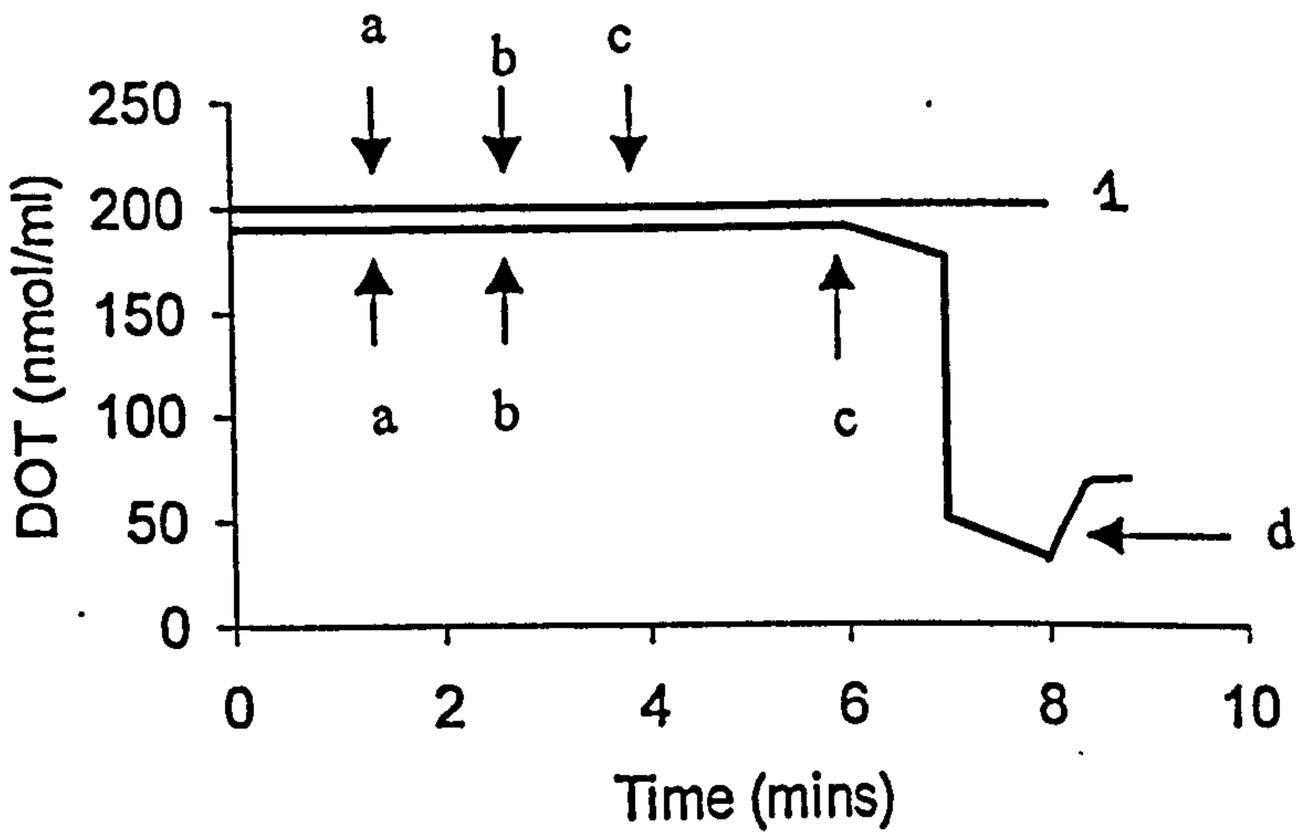
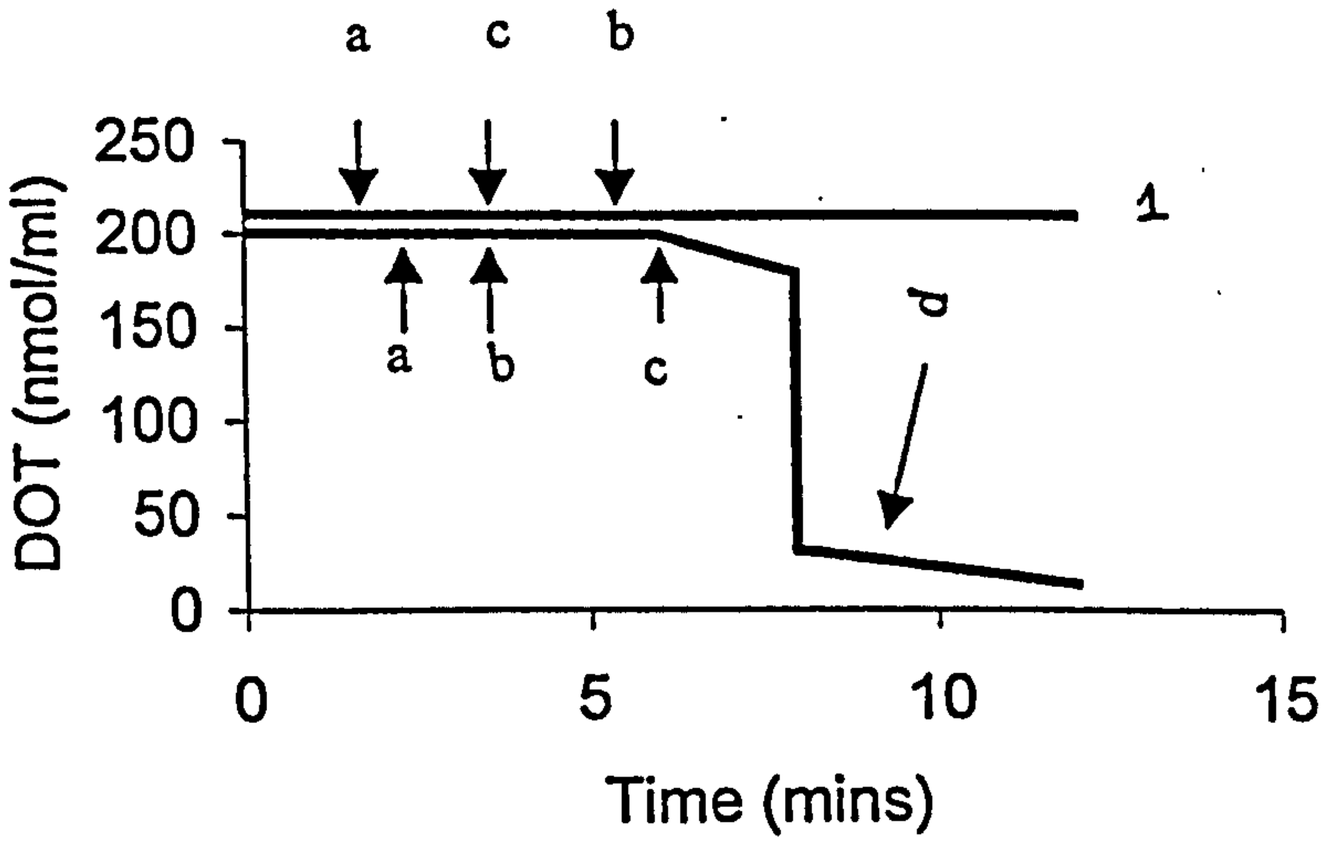


Figure 4.3 Production of H_2O_2 during NADH oxidation by *M. bovis* NCTC 10131(A) and 81B96 strain (B). Cell suspensions were lysed with Triton-X 100 at point 'a', GP (2.5 mM) was added at point 'b', and NADH (15 μM) was added at point 'c'. Catalase (5μl, 80 mg/ml) was added at point 'd' when oxygen uptake had ceased. Curve 1 is the control.

A



B

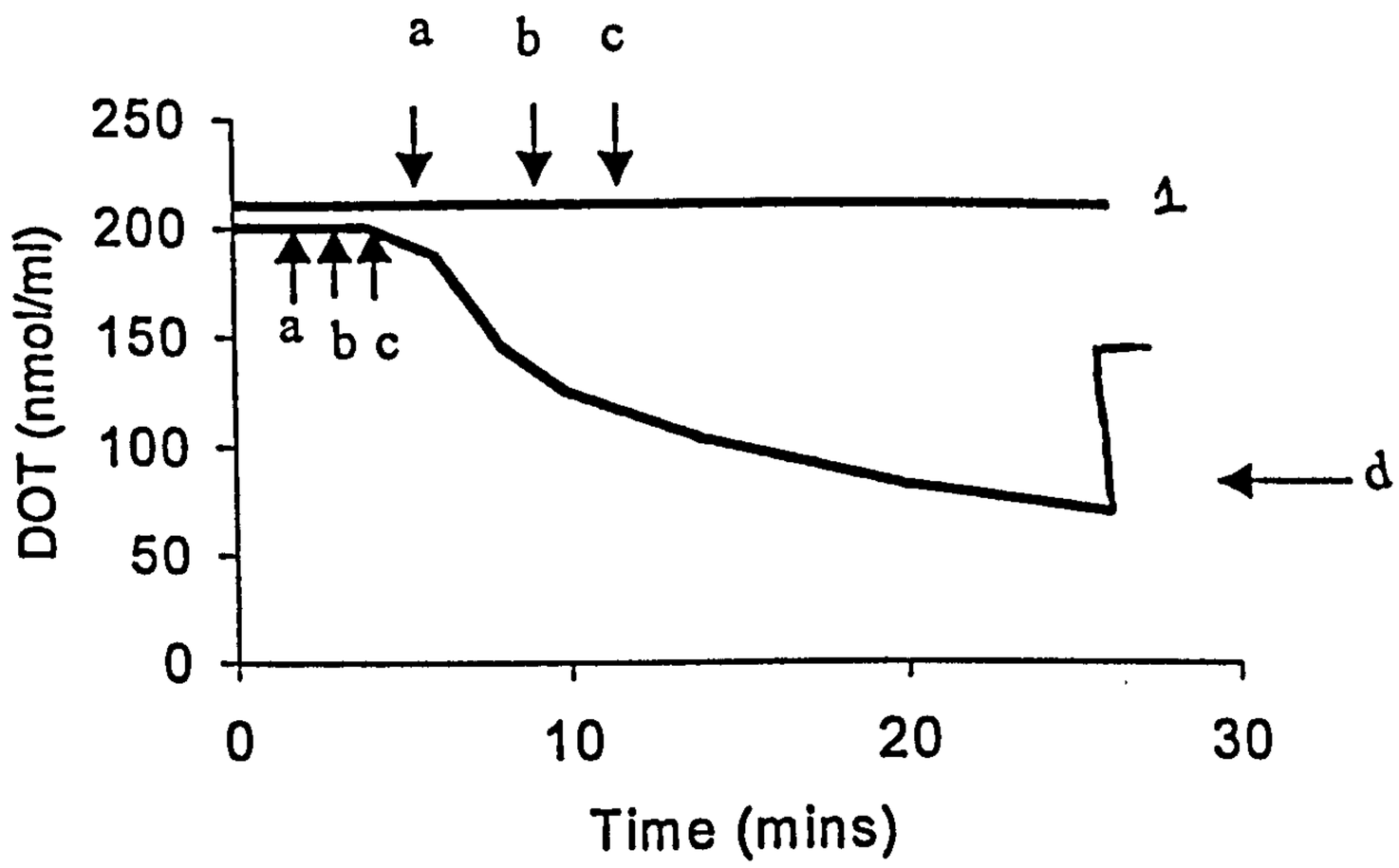
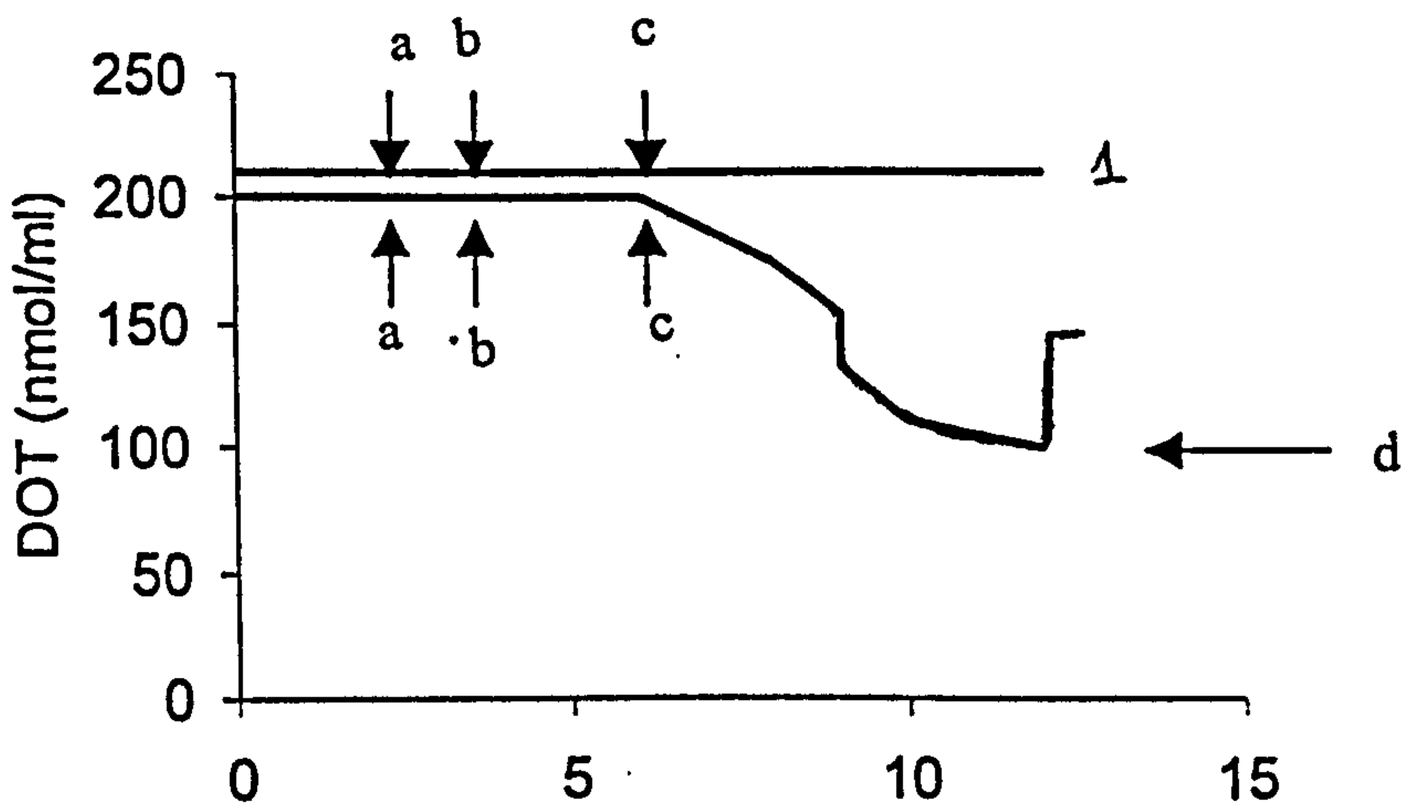


Figure 4.5 Production of H_2O_2 during NADH oxidation by *M.* ovine serogroup 11, 2D (A) and 50SR98 strain (B). Cell suspensions were lysed with Triton-X 100 at point 'a', GP (2.5 mM) was added at point 'b', and NADH (15 μM) was added at point 'c'. Catalase (5μl, 80 mg/ml) was added at point 'd' when oxygen uptake had ceased. Curve 1 is control.

A



B

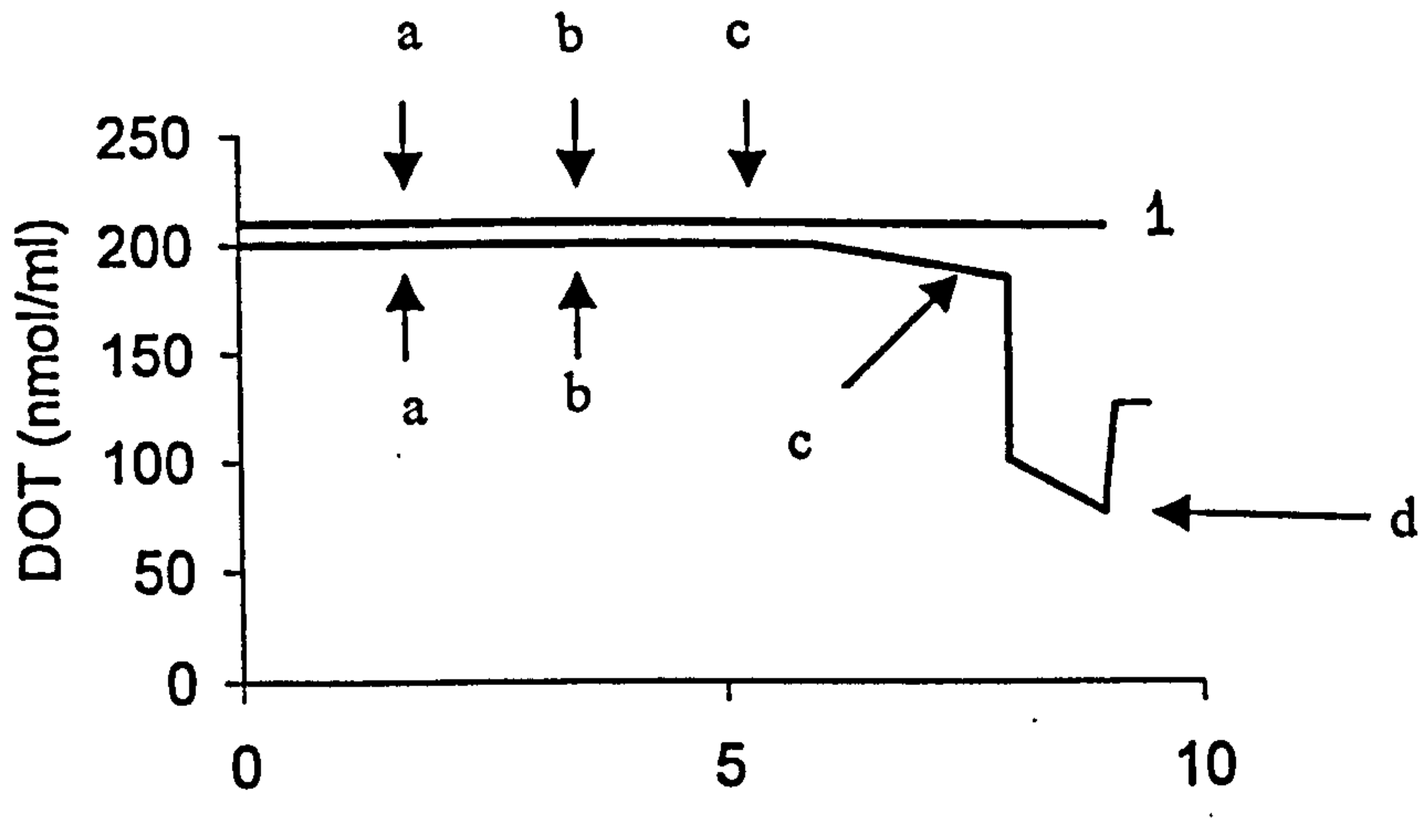
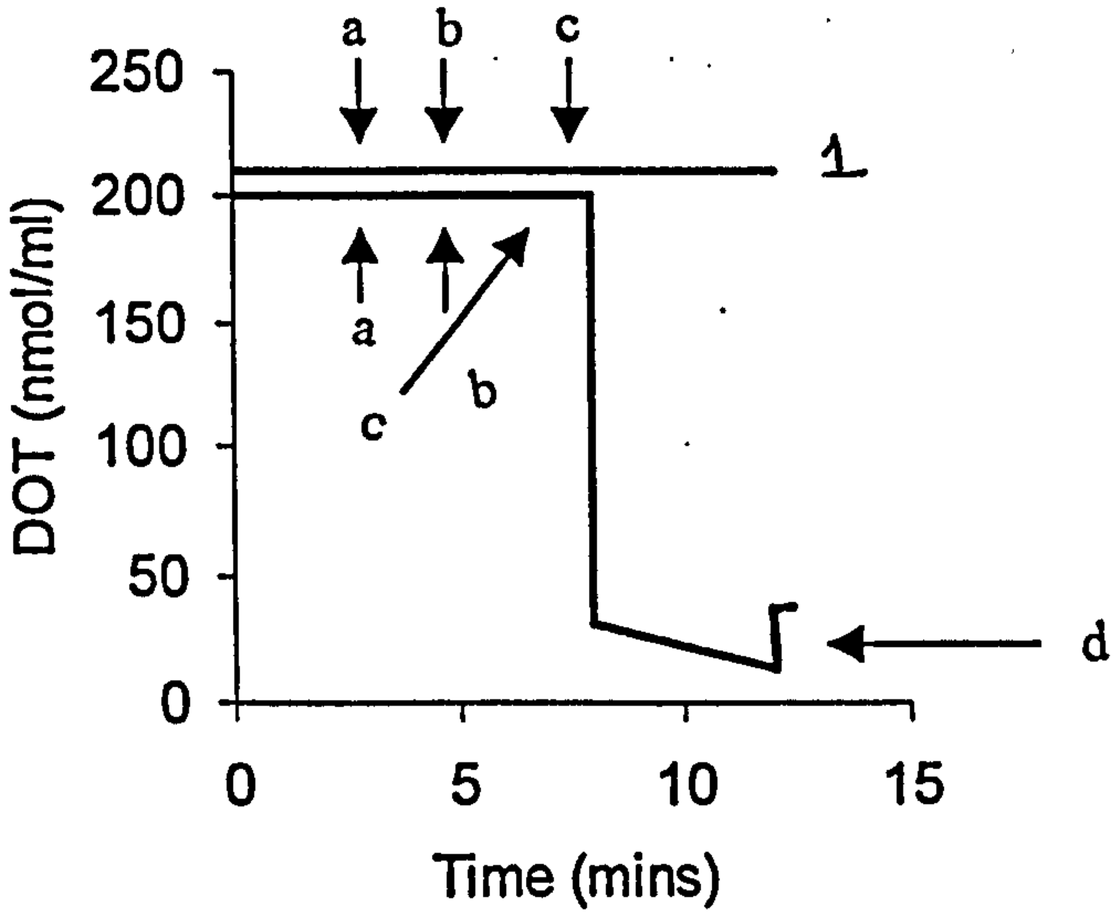
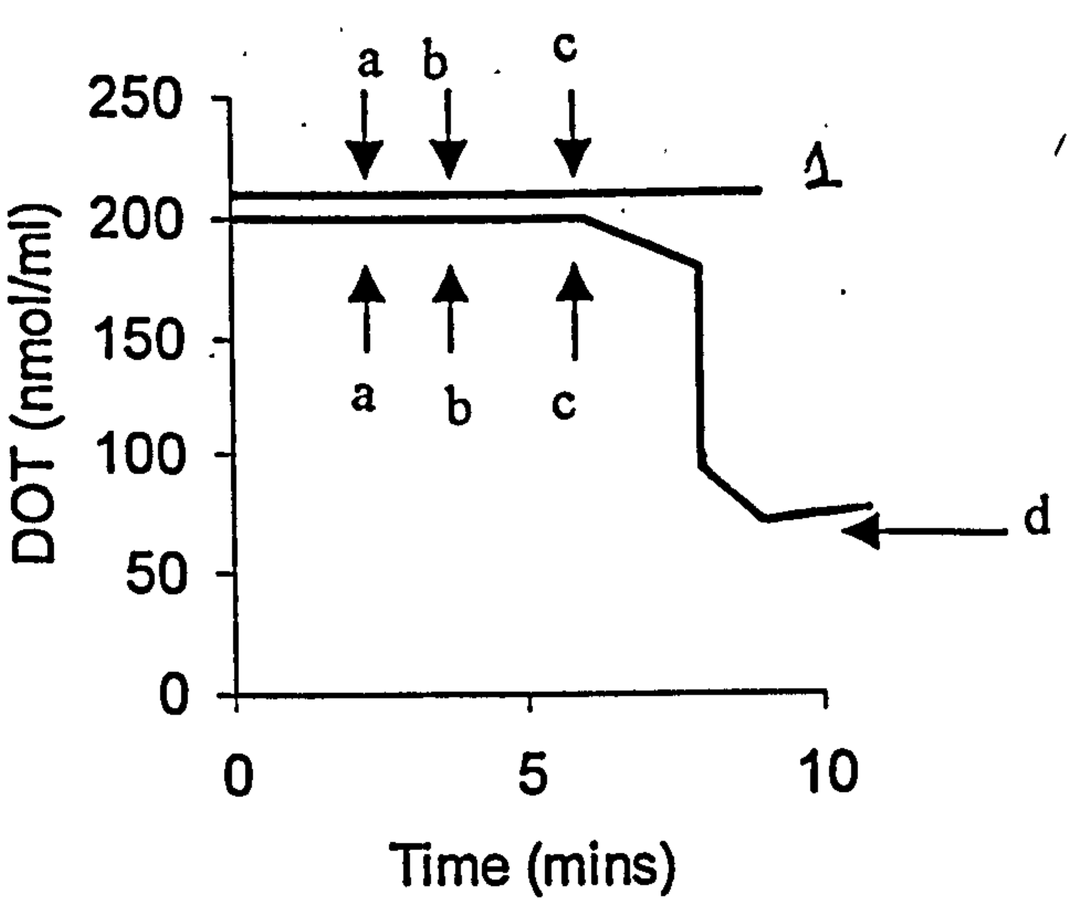


Figure 4.6 H_2O_2 production during NADH oxidation by *M. bovigenitalium* 434/81(A) and 398/87 strains (B). Cell suspensions were lysed with Triton-X 100 at point 'a', GP (2.5 mM) was added at point 'b', and NADH (15 µM) was added at point 'c'. Catalase (5µl, 80 mg/ml) was added at point 'd' when oxygen uptake had ceased. Curve 1 is the control.

A



B



In contrast, those species unable to oxidise glucose or organic acids, had a relatively low NADH oxidase activity (≥ 25 nmol/min/mg cell protein), which yielded approximately 1 mol H_2O_2 per mol of O_2 taken up. All of the strains in the present study oxidised organic acids and consistent with previous data, possessed relatively high NADH oxidase activity.

The high level of H_2O_2 production during the oxidation of NADH may be important in the pathogenicity of certain *M. agalactiae* and *M. bovis* strains. Kirkbride (1974) showed that high concentrations of mycoplasmas in an embryo culture could be responsible for inducing a toxic environment by producing metabolic products such as H_2O_2 resulting in the decreased fertilisation and embryo development rates. H_2O_2 may affect many diverse cellular functions and has, for example, been reported to cause peroxidation of polyunsaturated fatty acids, liberation of DNA bases and inactivation of enzymes (Halliwell and Gutteridge, 1995).

In addition, it is also possible that H_2O_2-yielding NADH oxidase activity might be selected against *in vitro*. Thus, *in vivo* and in low passage strains, H_2O_2 production from NADH oxidation might generally occur to a greater extent than is shown by analysis of most laboratory strains.

Typically, the strains in the present study produced little H_2O_2 during NADH oxidation, however some strains of *M. agalactiae* and *M. bovis* were exceptional and produced approximately 1 mol H_2O_2 per mol of O_2 taken up. These results were in line with a previous study of Abu-Groun (1992) who showed that the type strain of *M. c* subsp. *capripneumoniae* (F38) also produced 1 mol H_2O_2 per mol of O_2 although, other F38 strains produced little H_2O_2. Thus, it appears that there is substantial variation in the production of H_2O_2 by *M. agalactiae* and *M. bovis* strains. This may be important in relation to pathogenicity and could be of significance to epidemiological studies. Abu-Groun (1992) also reported two exceptional strains of *M. mycoides* subsp. *capri* (strains BQT and Pendik), which gave yields of 1 mol H_2O_2 per mol of O_2 taken up, during NADH oxidation. A possible explanation for the production of less than 1 mol H_2O_2 per mol of O_2 taken up during NADH oxidation, these strains may have two NADH oxidases. Two distinct NADH oxidase activities, have also been described in *Streptococcus mutans* (Higuchi *et al.*, 1993), a member of the *Lactobacillus* group to which mycoplasmas appear phylogenetically related (Maniloff, 1992). Although two

NADH oxidases activities were demonstrated in *M. pneumoniae* by Low and Zimkus (1973) only one NADH gene has been identified in the *M. genitalium* and *M. pneumoniae* genomes (Fraser *et al.*, 1995; Himmelreich *et al.*, 1996,1997). An alternative explanation is that most glucose or organic acid oxidising strains possess catalase activity. Although there is evidence for a mycoplasma thioredoxin reductase system, which might enable transfer of electrons from NAD(P)H to protein disulphide groups (Ben-Menachem *et al.*, 1997), there has been no convincing demonstration of catalase activity. Furthermore, catalase (and superoxide dismutase) genes have not been found in the *M. genitalium* and *M. pneumoniae* genomes.

4.2.2 H_2O_2 production from oxidation of L-α-glycerophosphate (GP)

None of the strains of the four species *M. agalactiae, M. bovis, M. bovigenitalium,* and *M.* ovine serogroup 11 oxidised GP at higher concentrations (2.5mM). GP oxidation consumes 1 mol of O_2 and yields 1 mol of dihydroxyacetone phosphate (DHAP) and 1 mol of H_2O_2 per mol of GP oxidised (Rodwell, 1967; Miles *et al.*, 1991). It has been shown in previous studies (Miles *et al.*, 1991; Taylor *et al.*, 1996; Houshaymi *et al.*, 1997) that during the oxidation of GP, lysed cells of glycerol-oxidising *Mycoplasma* strains produce approximately 1 mol H_2O_2 per mol oxygen taken up. This is consistent with the presence of a GP oxidase as suggested by Rodwell (1960). If GP was oxidised by a NAD^+-dependent dehydrogenase, the amount of H_2O_2 produced would be significantly less for most glucose-oxidising organisms because in these organisms, NADH oxidation generally leads to only low levels of H_2O_2 production (< 0.1 mol per mol O_2 taken up; Miles *et al.*, 1991; Taylor *et al.*, 1996). None of them oxidised glycerol at higher concentration (2.5 mM), therefore, the inability of these strains to oxidise glycerol may be attributed to a lack of GP oxidase activity, since they did possess glycerol kinase.

Cocks *et al.* (1985) reported that the production of H_2O_2 from GP, *via* NAD^+-linked GP dehydrogenase coupled to NADH oxidase, is also possible, and such a system may operate in ureaplasmas. Nevertheless, Rodwell (1967) demonstrated the absence of NAD^+-linked GP dehydrogenase activity in *M. mycoides* strain Y. This means that for organisms possessing GP oxidase activity, there is no metabolic route from the Emden-Meyerhoff pathway to GP, making them totally dependent upon an external source of glycerol for GP synthesis.

4.2.3 H_2O_2 production by whole cells metabolising pyruvate, isopropanol and NADH

The aim of this work was to assess the overall significance of the type of substrate on the quantity of H_2O_2 produced. H_2O_2 production during the metabolism of pyruvate (100 μM), isopropanol (100 μM) and NADH (15 μM) by whole mycoplasma cells equilibrated in Ringer-HEPES buffer was quantitatively determined by measurement of changes in DOT. Since the yield of H_2O_2 from NADH oxidation by lysed cells was variable, it was predicted that H_2O_2 formed during pyruvate oxidation would be similar (as glucose oxidation results in the net formation of 2 mol NADH per mol glucose). In addition, there would be expected to be a further small production of H_2O_2 from NADH oxidation, since isopropanol oxidation is accompanied by the net production of 2 mol NADH per mol isopropanol. The results obtained were as predicted. The yield of H_2O_2 by representative strains of *M. agalactiae, M. bovis, M. bovigenitalium* and *M.* ovine serogroup 11 was variable (Table 4.4). The NADH oxidation was also low compared to that of lysed cells. Whole cells of representative strains of *M. agalactiae* had low NADH oxidase activity (8-22 nmol/min/mg cell protein). Also, the quantities of H_2O_2 produced were low compared to the lysed cells (0-0.16 of H_2O_2 mol per mol oxygen taken up).

Similar results were seen when *M. bovis* whole cells were tested for NADH oxidase (13-14 nmol/min/mg cell protein). H_2O_2 production was also less compared to lysed cells (0-0.46 of H_2O_2 mol per mol of oxygen taken up). In contrast the type strain *M. bovis* NCTC 10131 did not produce H_2O_2 during oxidation of NADH in lysed cells. Whole cells of *M. bovis* type strain also did not produce H_2O_2 and these results agreed with the previous data (4.2.1). Representative strains of *M.* ovine serogroup 11 whole cells also had low NADH oxidase activity (8-17 nmol/min/mg cell protein) and the quantities of H_2O_2 produced were low compared to the lysed cells (0-0.14 of H_2O_2 mol per mol oxygen taken up; Table 4.4).

NADH oxidation by whole cells of *M. mycoides* SC was also low compared to lysed cells (166 nmol/min/mg cell protein) (Table 4.4). The addition of NADH to whole cells of all these strains resulted in relatively low rates of oxygen uptake. This was expected as NADH oxidase activity in mycoplasmas is reported to be cytoplasmic (Pollack, 1975). This might be a possible explanation for the low NADH oxidase activity and

when the cells were lysed, enzyme was released and may have shown high rates of NADH oxidation. However, rates of NADH oxidation were very high in lysed rather than in whole cells (Table 4.1, 4.2 and 4.3). It is also possible that NADH oxidation by whole cells might have been due to the presence of autonomously lysed cells. None of the strains were able to oxidise GP, and these results were consistent with those in Section 4.2.2.

Whole cells of *M. agalactiae, M. bovis,* and *M.* ovine serogroup 11 were tested for the production of H_2O_2, which was measured from changes in DOT when catalase was omitted during the washing of cells. It was shown that the production of H_2O_2 by whole cells of *M. agalactiae* during the oxidation of pyruvate (0-0.58 of H_2O_2 mol per mol oxygen taken up) and isopropanol (0-0.31 of H_2O_2 mol per mol of oxygen taken up) was low. H_2O_2 production by whole cells of *M. bovis* during the oxidation of pyruvate and isopropanol was variable (pyruvate, 0.26-1.2 and isopropanol, 0.24-036 of H_2O_2 per mol oxygen taken up). These values also showed variation compared to the lysed cells.

Whole cells of *M.* ovine serogroup 11 showed variable H_2O_2 production: during the oxidation of pyruvate it was 0.33-0.69 mol of H_2O_2 per mol oxygen taken up and during the oxidation of isopropanol it showed variations of 0.21-0.74 of H_2O_2 per mol of oxygen taken up (Table 4.5). The production of H_2O_2 during the oxidation of pyruvate and isopropanol was shown to be very low compared to lysed cells. These results confirmed the original prediction.

In the mycoplasma strains that oxidise glucose to acetate relatively large quantities of NADH will be formed, whereas in *M. fermentans* and *M. canis* which apparently convert glucose to lactate under aerobic and anaerobic conditions there will be little NADH formation. The accumulation of H_2O_2 during mycoplasma metabolism will be dependent on the nature of the substrate available (glucose, pyruvate, alcohols), on the metabolic pathways of the mycoplasma species and on the regulation of the H_2O_2-producing enzymes. H_2O_2 production might be selected against during growth *in vitro* (Wadher *et al.*, 1990).

Table 4.1 NADH oxidase, GP oxidase activities and H_2O_2 production during NADH, GP oxidation by the lysed cells of *M. agalactiae* (European strains).

Strain code	**NADH oxidation (nmol/min/mg cell protein)**	**H_2O_2 production (mol per mol of O_2 taken up)**
NCTC 10123	928	0.07
2123/91	857	0.14
723/93	359	0.29
499/93	548	0
1070/93	437	0.08
1209/93	410	0.06
471/93	353	0.05
453/94	40	1.03
101/94	36	1.21
730/97	376	0.02
314/97	331	0.08
423/98	374	0.14
4400/99	475	0.12
2245/99	635	0
1536/99	796	0.06
LF/00	569	0.05
4a	819	0.16
10a	777	0.09
11b	798	0.08
6gb	840	0.07
A.vet	525	0.09
3328	735	0.1

GP, not oxidised

Table 4.2 NADH oxidase, GP oxidase activities and H_2O_2 production during NADH, GP oxidation by the lysed cells of *M. bovis.*

Strain code	**NADH oxidation (nmol/min/mg cell protein)**	**H_2O_2 production (mol per mol of O_2 taken up)**
NCTC 10131	329	0
79B96	93	0.59
81B96	139	0.75
82B96	140	0.78
119B96	145	1.02
193B96	62	0.90
67M98	122	0.72
135B99	16	0.49
136B99	37	1.10
137B99	52	0.31
139B99	124	0.29
142B99	165	0.14
156B99	157	0.20
5B00	94	0.62
10B00	22	0.30
12B00	95	0.20

GP, not oxidised

Table 4.3 NADH oxidase, GP oxidase activities and H_2O_2 production during NADH and GP oxidation by the lysed cells of *M.* ovine serogroup 11 and *M. bovigenitalium.*

Strain code	**NADH oxidation (nmol/min/mg cell protein)**	**H_2O_2 production (mol per mol of O_2 taken up)**
2D	171	0.44
48SR98	663	0.12
50SR98	1412	0.16
52SR98	491	0.21
3SR99	399	0.09
47SR99	645	0.06
48SR99	785	0.04
52SR99	608	0.11
95SR99	796	0.07
96SR99	774	0.13
126SR99	675	0.06
129SR99	637	0.07
M. bovigenitalium NCTC 10122	605	0.13
57B00	244	0.07
398/87	708	0.07
434/81	1039	0.05

GP, not oxidised

4.2.4 Effect of *in vitro* serial passage of *M. bovis* 119B96 strain on production of H_2O_2.

There was substantial variation in the production of H_2O_2 by *M. agalactiae* and *M. bovis* strains. *M. bovis* strain 119B96 produced 1 mol of H_2O_2 per mol of O_2 taken up. Gabridge *et al.* (1985) have shown that production of H_2O_2 might be an important factor in relation to mycoplasma pathogenicity. Baeuerle and Henkel (1994) reported H_2O_2 is assumed to damage the host by directly impairing tissue cell or inducing gene expression in the host e.g. proinflammatory genes via activation of NF-kB. Several other reports suggested that H_2O_2 which is released in certain mycoplasmas, is a powerful mediator of cell injury (Miles *et al.*, 1991; Tryon and Baseman, 1992). Wadher *et al.* (1990) showed that *M. mycoides* subsp. *mycoides* SC had lost the H_2O_2 producing enzyme, L-α- glycerophosphate oxidase during *in vitro* passage. The major aim of this work was to serially passage this strain and check the effect of *in vitro* passage on H_2O_2 production. Serial passage was performed in broth medium, subculturing every 48-72 hours.

In this experiment a high H_2O_2 producing *M. bovis* strain was serially passaged and production of H_2O_2 was measured from increase in DOT. NADH activity was expressed as initial rates of oxygen uptake. H_2O_2 production was monitored at 0, 5th, 20th, 50th, 120th, 140th, and 200th passage. All passages of *M. bovis* 119B96 strain had a variable NADH oxidase activity (60-210 nmol/min/mg cell protein). The quantities of H_2O_2 produced decreased with increasing passage. At zero passage H_2O_2 was 1.02 mol per mol of oxygen taken up; after the 50th passage the amount of H_2O_2 was reduced 50% (0.52 mol per mol of oxygen taken up). H_2O_2 produced after the 100th passage was 15% (0.16 mol per mol of oxygen taken up) and after the 200th passage H_2O_2 produced was 7% (0.07 mol per mol of oxygen taken up) (Table 4.6 and Figure 4.7).

Cells of the 200th high passage *M. bovis* strain 119B96 were also tested for substrate oxidation by measurement of oxygen uptake, detecting changes in DOT described in Section 2.11.1. High passaged *M. bovis* strain 119B96 oxidised substrates similar to the parent strain. It oxidised organic acids such as L-lactate, 2-oxobutyrate and pyruvate, and isopropanol even at low concentrations (<25μM). It also oxidised ethanol and propanol at rates consistent with the parent strain. High passage strain 119B96 was also unable to oxidise sugars (N-acetyl glucosamine, fructose, glucosamine, glucose,

maltose, mannose, and sucrose; 2.5 mM) glycerol (2.5 mM) and the organic acids (fumarate, malate and 2-oxoglutarate; 2.5mM).

The rates of oxygen uptake in the presence of L-lactate and 2-oxobutyrate were 60 and 57 % of the rate with pyruvate respectively. The rates of oxygen uptake in the presence of isopropanol, ethanol, and propanol were 423, 14 and 25 % respectively for passage zero *M. bovis* strain 119B96. The rates of oxygen uptake (% rate of pyruvate) were, in the presence of L-lactate 150-159%, 2-oxobutyrate 76-100%, isopropanol 555-1093%, ethanol 42-47% and propanol 15-17%, for *M. bovis* 119B96 passage 100 and 200 (Table 4.7). This showed that *in vitro* passage did not affect substrate oxidation, as the patterns of substrate oxidation were same. *M. bovis* 119B96 passage 100 and 200 showed identical patterns of substrate oxidation to that of the parent strain however rates of oxidation were high compared to the parent strain, the reason for which is unclear. This showed that the rates of substrate oxidation were not reduced when cells were passaged.

Dyson and Smith (1976) showed that serial passage of *M. mycoides* subsp. *mycoides* led to a striking reduction in the ability of strains to produce bacteraemia in mice. They found broth culture passage readily attenuated strains but no detectable mouse virulence was seen as a result of passage in egg and mice. These results illustrate the potential value of *in vitro* passage, for the development of vaccine for *M. bovis* infections. It is perhaps arguable that the apparently low virulence of the African vaccine strain T_1, which has been passage over 40 times in eggs (Provost *et al.*, 1987), has more to do with the site of inoculation (the tail tip) than with induced attenuation. Somerville *et al.* (2002) reported that *S. aureus* undergoes significant phenotypic and genotypic changes during serial passage.

They also reported that serial passage of bacteria can result in genomic diversity of derivative strains relative to the parental strain due to single nucleotide changes, changes in repetitive DNA, recombination and insertion and deletion events. Aconitase specific activity decreased over time during *in vitro* passage. Aconitase is a bifunctional protein and has been postulated to be a virulence factor regulator (Somerville *et al.*, 1999).

These results are also in agreement with Thorns and Boughton (1980) who reported that the *M. bovis* strain that had been passaged more than 60 times were markedly less virulent than the same or different strains with less passage.

Table 4.4 H_2O_2 production and NADH and GP oxidation by whole cells of *M. agalactiae* (European strains), *M. bovis, M.* ovine serogroup11 and *M. mycoides* SC.

Strain code	**NADH oxidation (nmol/min/mg cell protein)**	**H_2O_2 production (mol per mol of O_2 taken up)**
M. agalactiae NCTC 10123	18	0.08
2123/91	9	0.16
1070/93	15	0.04
453/94	18	0.16
499/94	9	0
432/98	13	0
4400/99	8	0.16
LF/00	22	0
M. bovis NCTC 10131	14	0
79B96	13	0
5 B00	26	0.13
10B00	13	0.46
M. ovine serogroup 11 95SR99	17	0.14
126SR99	8	0
129SR99	13	0
M. mycoides SC clone 6	166	0

Table 4.5 Production of H_2O_2 and oxygen uptake during oxidation of pyruvate and isopropanol by whole cells of *M. agalactiae*, *M. bovis*, *M.* ovine serogroup 11.

Strain code	**Pyruvate oxidation (nmol/min/ mg cell protein)**	**H_2O_2 production mol per mol of O_2 taken up)**	**Isopropanol oxidation (nmol/min/mg cell protein)**	**H_2O_2 production mol per mol of O_2 taken up)**
M.agalactiae NCTC 10123	38	0.45	75	0.10
453/94	47	0.58	188	0.25
432/98	38	0.37	28	0.31
LF/00	51	0	67	0
M. bovis NCTC 10131	46	0	309	0
81B96	27	1.2	177	0.27
82B96	40	0.62	199	0.25
119B96	56	0.26	126	0.36
5B00	31	0.66	265	0.24
*M.*ovine serogroup11 95SR99	38	0.69	21	0.74
96SR99	31	0.68	73	0.21
126SR99	25	0.33	40	0.42
129SR99	42	0.47	33	0.29

GP, not oxidised

The four low passage strains of *M. bovis* produced severe inflammatory response in the mammary glands while high passaged *M. bovis* showed reduced virulence for mammary glands.

Anderson *et al.* (1976) found marked differences in virulence between the avirulent vaccine strain KH_3J and the highly virulent Gladysdale strain of *M. mycoides* subsp. *mycoides.* It is assumed that the strains passaged many times in liquid medium lose their virulence. These mutants decreased the production of secreted virulence factor (H_2O_2) and increased the growth yield. These mutants may be used as live attenuated *M. bovis* vaccine for cattle. SDS-PAGE and RFLP analysis of these mutants showed marked differences (Chapter 6). Wayne *et al. (*1990) showed that rats vaccinated with killed *Mycoplasma pulmonis* organisms were only partially protected from challenge, and viable wild type *M. pulmonis* organisms were more protective than killed organisms. Live vaccines may also stimulate long lasting immunity because the organism multiplies in the host and thus continuously stimulates the immune system both locally and systemically and another property of live vaccine is genetic stability (Greenberg *et al.*, 1974).

4.2.5 Detection of glycerol kinase activity

The representative strains of *M. agalactiae* showed glycerol kinase activity 22 and 64 nmol/min/mg cell protein. The glycerol kinase activity for *M. bovis* strains tested was 49-67 nmol/min/mg cell protein and glycerol kinase activity for *M.* ovine serogroup 11 was between 8-25 nmol/min/mg cell protein. The strains tested were all glycerol negative (Table 4.8). It showed that failure of all *M. agalactiae*, *M. bovis*, *M. bovigenitalium* and *M.* ovine serogroup 11 strains to oxidise glycerol was not due to a lack of glycerol kinase activity.

Glycerol is phosphorylated by glycerol kinase to GP in a reaction requiring ATP. Glycerol kinase activity has been reported in *M. mycoides* (Wadher *et al.*, 1990), but direct evidence for the existence of a gene analogous to the putative glycerol kinase gene *glp*K of *M. genitalium* is still lacking (Fraser *et al.*, 1995). Glycerol is metabolised via G3P oxidase (α-GPO) in a reaction which yields 1 mol of H_2O_2 per mol of G3P oxidised.

Table 4.6 Effect of passage on H_2O_2 production by *M. bovis* strain 119B96. NADH oxidase activity and H_2O_2 production during oxidation of NADH by lysed cells of *M.bovis* 119B96 strain.

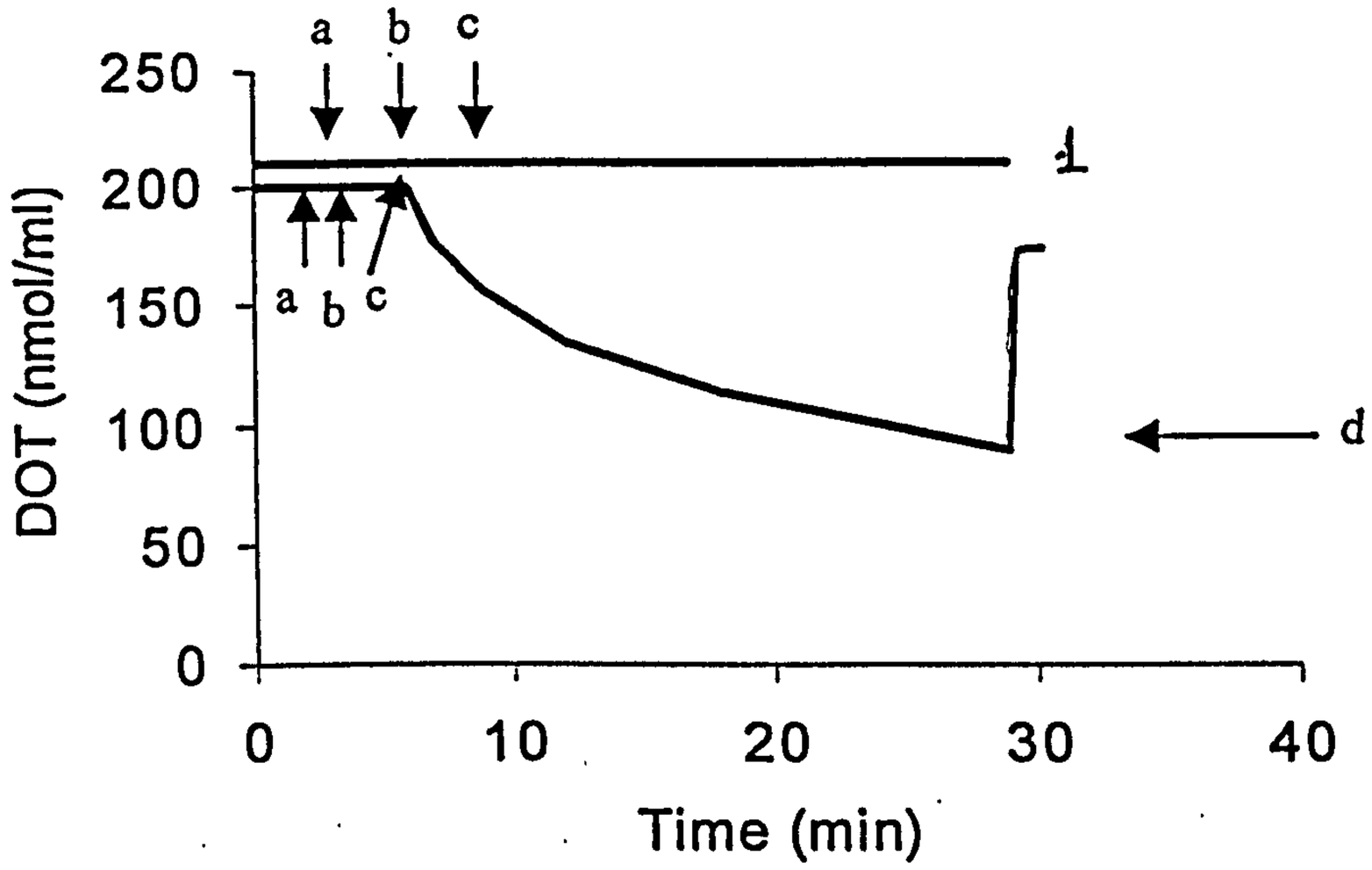
Passage number	**NADH oxidation (nmol/min/mg cell protein)**	**H_2O_2 production (mol per mol of O_2 taken up)**
0	145	1.02
5	126	0.86
20	264	0.81
50	60	0.52
100	112	0.16
120	115	0.17
140	210	0.11
200	175	0.07

Table 4.7 Substrate oxidation by zero, medium and high passage strains of *M. bovis* 119B96.

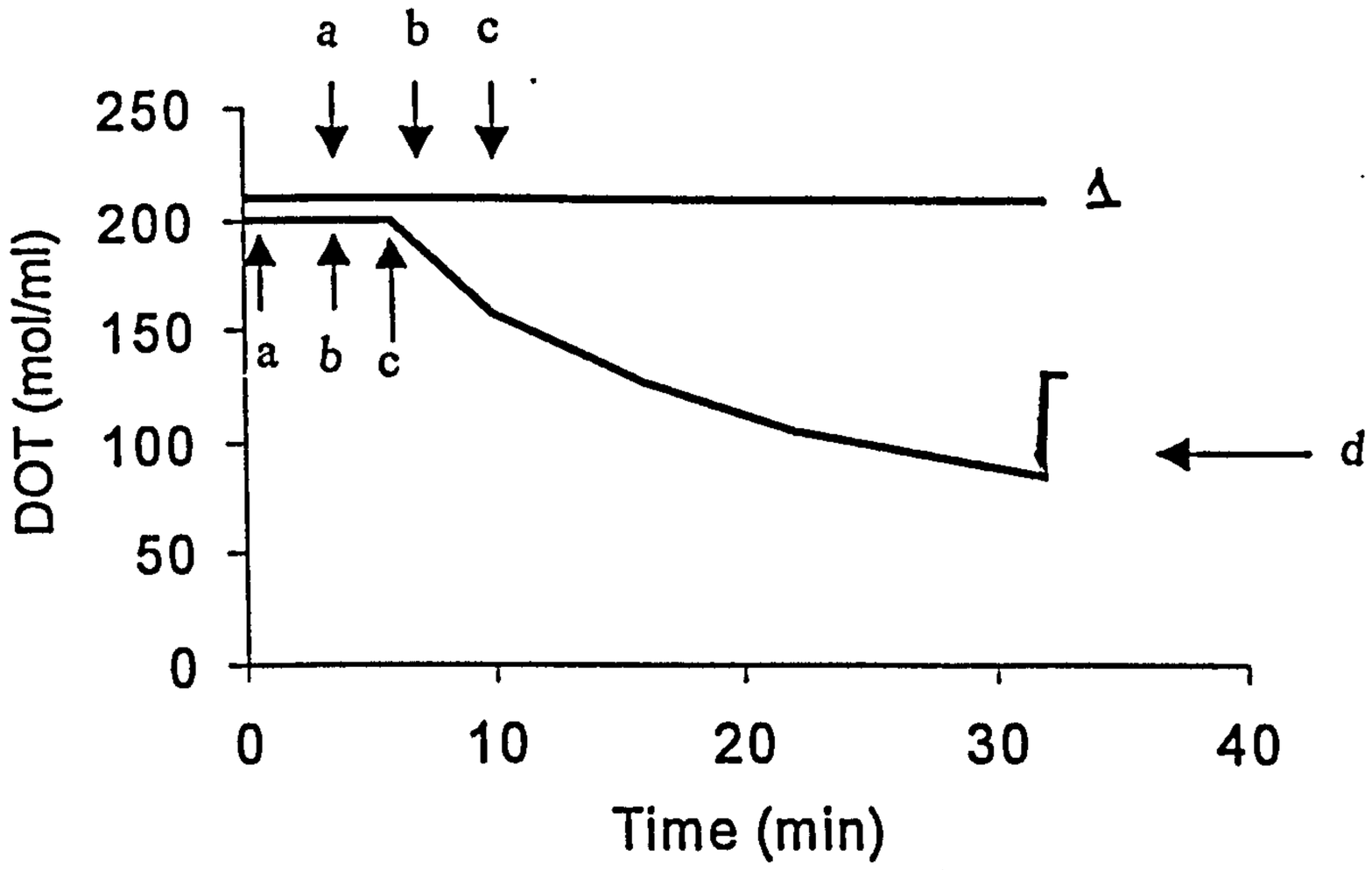
Passage number	**(Relative rates of oxygen uptake (% of pyruvate)**					
	L-lactate	**2-oxobutyrate**	**pyruvate**	**isopropanol**	**propanol**	**ethanol**
0	60	57	100	423	14	25
100	159	76	100	1093	15	42
200	150	100	100	555	17	47

Figure 4.7 Production of H_2O_2 during NADH oxidation by *M. bovis* strain119B96 low passage (A) passage 50 (B) passage 200 (C). Cell suspensions were lysed with Triton-X 100 at point 'a', GP (2.5 mM) was added at point 'b', and NADH (15 μM) was added at point 'c'. Catalase (5μl, 80 mg/ml) was added at point 'd' when oxygen uptake had ceased. Curve 1 is control.

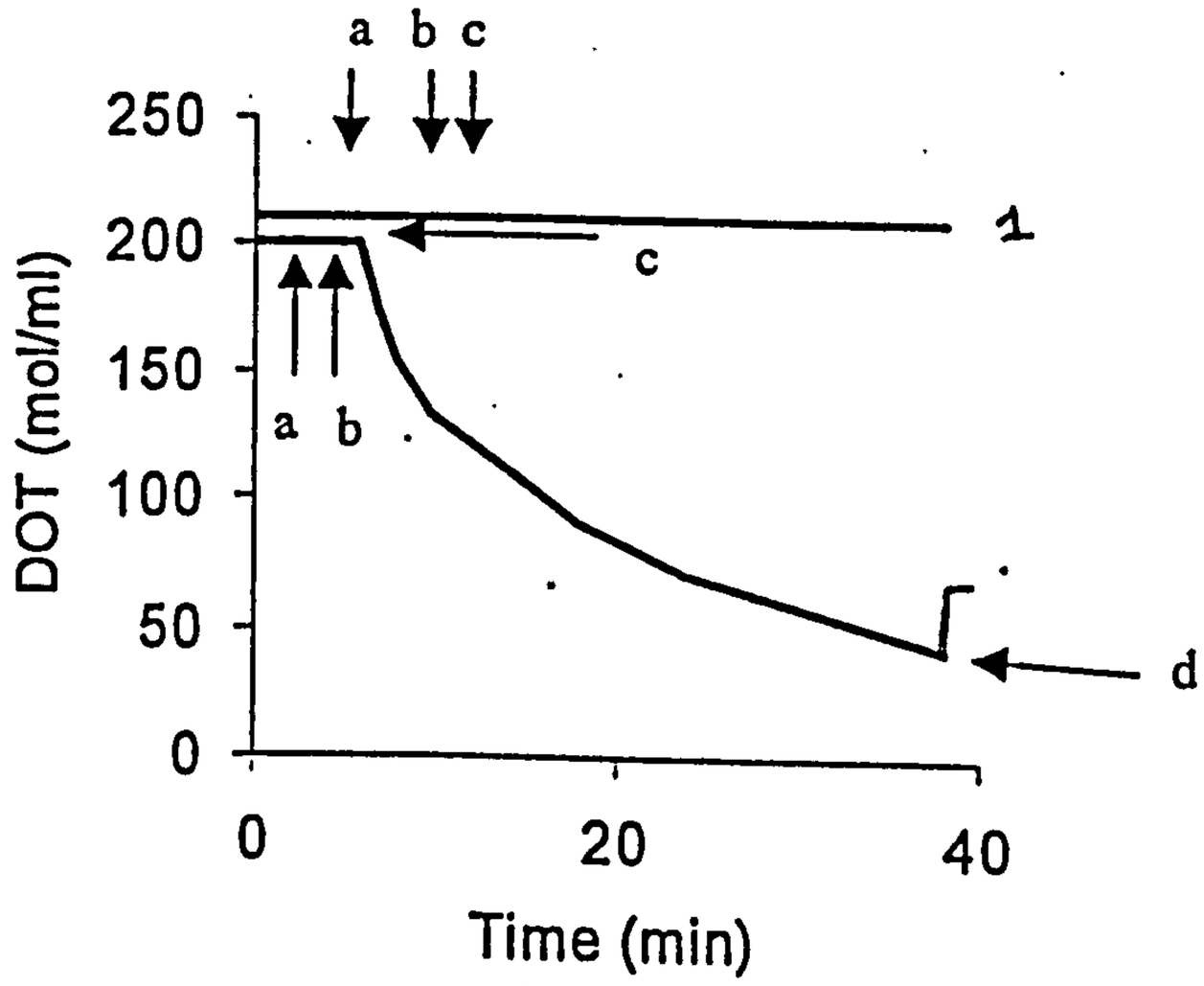
A



B



C



This previously led to speculation that the lack of the H_2O_2 producing enzyme α-GPO was responsible for the reduced pathogenicity of some *M. mycoides* subsp. *mycoides* SC strains (Wadher *et al.*, 1990; Miles *et al.*, 1991).

Vilei and Frey (2001) showed that the lack of H_2O_2 production by certain strains of *M. mycoides* subsp. *mycoides* was due to lack of an active glycerol uptake system encoded on the *gts* ABC operon. Glycerol may enter mycoplasmas by passive diffusion (Romijin *et al.*, 1972) and eventually by a putative glycerol uptake facilitator gene (*glp*F) product, as is found in *M. genitalium* (Fraser *et al.*, 1995). The amount of glycerol that can be taken up by these two systems must be considerably smaller than that incorporated by the active uptake system specified by GtsABC. It is known that mycoplasmas lack hexokinase, which is essential for the first step in the glycolytic pathway (Pollack *et al*, 1997). This absence is balanced by the presence of a sugar phosphotransferase, which traps sugars from the environment and phosphorylates them directly during import into cells.

For *M. agalactiae* and *M. bovis*, the glycerol kinase activity was high and comparable to that for *M.* ovine serogroup 11 strains, however None of the strains of the three species oxidised glycerol. Glycerol is phosphorylated by glycerol kinase to GP and is presumably required by all mycoplasmas for the synthesis of triglycerides. The results were as expected; glycerol kinase activity was detectable in all *M. agalactiae, M. bovis, M.* ovine serogroup11 strains tested which were all negative for glycerol-oxidation. PEP-linked glycerol kinases have not previously been reported in any organism. It appears possible that glycerol might be a substrate for a PEP:PTS-linked uptake system. In *M. pneumoniae*, a mannitol-specific enzyme IIA has been tentatively identified on the basis of genome sequence (Himmelreich *et al.*, 1997). Enzyme IIA catalyses the phosphorylation of substrate bound to substrate-specific enzyme IIB, which are located within the cell membrane. Mannitol is a six-carbon sugar alcohol and thus shares structural similarities with glycerol. Although an enzyme IIB for mannitol has not yet been identified in *M. pneumoniae*, it is conceivable that mannitol is a PEP:PTS substrate. Thus, it may be that a mannitol-specific PTS exists in certain strains of the *M. mycoides* cluster, and that its substrate specificity is sufficiently broad to enable glycerol uptake and phosphorylation.

4.3 H_2O_2 production and NADH oxidation by lysed cells of *M. mycoides* subsp. *mycoides* SC strains.

All of the test strains were fermentative mycoplasmas such as *M. mycoides* subsp. *mycoides* SC. Washed cells of all the strains were prepared as previously described in Section 2.7 except catalase was omitted and H_2O_2 production was monitored from increase in DOT. All *Mycoplasma mycoides* strains studied possess high NADH oxidase activities, which resulted in the variable production of H_2O_2 (0.04-1.05 mol of H_2O_2 per mol of O_2 taken up). All these strains were isolated from experimental animals and were subsequently cloned three times. All these strains showed high NADH oxidase activity (430-1095 nmol/min/mg cell protein) and produced variable H_2O_2 (Table 4.9).

African strain SH9 was different from the European SC strains, as it oxidised GP (76 nmol/min/mg cell protein), and NADH (553 nmol/min/mg cell protein). H_2O_2 production by this strain from L-α-glycerophosphate oxidation was 0.56 mol of H_2O_2 per mol O_2 taken up and from NADH oxidation 0.15 mol of H_2O_2 per mol O_2 taken up. African strain SC SH9 showed consistent NADH oxidation with European strains but showed a difference in GP oxidation as European strains were unable to oxidise GP. These results were in agreement with Houshaymi *et al.* (1997), who found that European strains of *M. mycoides* were unable to oxidise glycerol and GP.

Miles *et al.* (1991) and Taylor *et al.* (1996) found that the species which oxidise glucose (to acetate) had relatively high NADH oxidase activity and yielded only small quantities of H_2O_2. African strain SH9 oxidised GP and produced <1 mol of H_2O_2 per mol oxygen taken up. These results were in contrast to Rodwell (1967) and Miles *et al.* (1991) who found that the oxidation of GP results in the production of 1 mol of H_2O_2 per mol of oxygen taken up. If GP was oxidised by a NAD^+-dependent dehydrogenase, the amount of H_2O_2 produced would be significantly less for glucose oxidising mycoplasmas.

All these strains were glucose-oxidising but were unable to oxidise glycerol possibly due to lack of glycerol oxidising enzymes.

Table 4.8 Glycerol kinase activity in *M. agalactiae, M. bovis*, and *M.* ovine serogroup 11 strains.

Strain	**Glycerol oxidation (nmol/min/mg cell protein)**	**Glycerol kinase activity (nmol/min/mg cell protein)**
Mycoplasma agalactiae NCTC 10123	Nd	22
423/98	Nd	47
2245/99	Nd	64
1536/99	Nd	39
LF/00	Nd	31
Mycoplasma bovis NCTC 10131	Nd	49
82B96	Nd	43
119B96	Nd	67
139B99	Nd	39
5B00	Nd	55
Mycoplasma ovine serogroup 11 48SR 99	Nd	8
95SR99	Nd	25
126 SR 99	Nd	13
129 SR 99	Nd	18

Nd, not detected

Table 4.9 H_2O_2 production and NADH oxidation by lysed cells of *M. mycoides* SC.

Strain code	GP oxidation (nmol/min/mg cell protein)	NADH oxidation (nmol/min/mg cell protein)	H_2O_2 production (mol per mol of O_2 taken up)
clone1	Nd	441	0.17
clone2	Nd	504	0.12
clone3	Nd	436	0.12
clone4	Nd	661	0.18
clone5	Nd	451	0.16
clone 6	Nd	1095	0.04
clone 7	Nd	581	0.90
clone 8	Nd	707	1.05
clone 9	Nd	455	0.93
clone 10	Nd	557	0.87
clone 11	Nd	430	0.98
clone 12	Nd	623	0.85
clone 13	Nd	546	0.86
clone 14	Nd	511	0.84
SH9	76	553	0.15 0.56♣

♣ H_2O_2 production (mol/mol of O_2 taken up) during the oxidation of GP.

NADH was oxidised at high rates by all *M. mycoides* strains but the rate of H_2O_2 production was consistently low except clones 7-14 which produced approximately 1 mol of H_2O_2 per mol of oxygen taken up (Table 4.9). These results were in agreement with Abu-Groun (1992) who found that among the *M. mycoides* cluster, three strains gave approximately 1 mol H_2O_2 per mol O_2 taken up. None of the European strains were able to oxidise GP at higher concentrations (2.5 mM), indicating that they lacked glycerol oxidising enzymes and may be less virulent. They also showed that the glucose-oxidising species, *A. laidlawii*, apparently possessed only a single NADH oxidase activity, which gave a yield of 1 mol of H_2O_2 per mol of NADH oxidised.

4.4 Conclusions

The NADH oxidase activity was shown to be very high in all the strains and they produced low H_2O_2 per mol of O_2 taken up during NADH oxidation. *M. agalactiae* and *M. bovis* (four strains) produced approximately 1 mol of H_2O_2 per mol of O_2 taken up. All *M. mycoides* SC strains showed low H_2O_2 per mol of O_2 taken up and NADH oxidase activity was very high except for eight strains, which produced approximately 1 mol H_2O_2. The high production of H_2O_2 may be important in their pathogenicity and may also be significant in epidemiological studies.

None of the mycoplasma strains studied oxidised glycerol and GP with the exception of African *M. mycoides* strain SH9, which oxidised glycerol and GP. Their inability to oxidise glycerol may be attributed to a lack of GP oxidase activity, since they possess glycerol kinase activity. *M. mycoides* SC strain SH9 showed high levels of GP oxidase activity (76 nmol/min/mg cell protein) and GP oxidation led to the production of H_2O_2, so it is possible that the African strain SH9 may be more pathogenic than other *M. mycoides* strains tested, which were unable to oxidise GP.

Mollicutes are known to produce active oxygen species, and particularly H_2O_2 during growth. In this chapter it has been confirmed that H_2O_2 production accompanies substrate oxidation by *M. agalactiae*, *M. bovis* and *M.* ovine serogroup 11. H_2O_2 production by whole cells of the representative strains during oxidation of pyruvate, isopropanol and NADH was also determined. The aim was to assess the overall significance of the substrate type on the quantity of H_2O_2 produced. The rates of H_2O_2 production from pyruvate, isopropanol and NADH oxidation were low. NADH oxidase in whole cells was also consistently low compared to lysed cells. In contrast H_2O_2

production during the oxidation of NADH by lysed cell was variable. However, H_2O_2 produced by whole cells during the oxidation of isopropanol might be significant in pathogenicity. This suggested that the ability to produce H_2O_2 might be restricted to those organisms (oxidising glucose or organic acids) which generate large quantities of NADH during metabolism, otherwise production of H_2O_2 might result in autonomous cell death. It has been confirmed that H_2O_2 is produced during substrate (pyruvate or isopropanol) oxidation by non-fermentative and non-arginine hydrolysing mycoplasmas.

M. bovis strain 119B96 which produced highest H_2O_2 (1 mol) was serially passaged in broth culture and showed the quantities of H_2O_2 produced decreased with increasing passage. The decrease in H_2O_2 may be because the high passage strain have lost its NADH oxidase activity for H_2O_2 production. This is a very significant finding because the reduced production of H_2O_2 by a high passage strain is very important in terms of pathogenicity. The high passage strain might be less virulent than the parent strain and therefore be a good candidate for attenuated live vaccine for *M. bovis* infection. Live vaccines are superior to killed vaccines as they provide both quantitatively and qualitatively better protective immunogens as chemical and physical procedures used to prepare killed vaccines may destroy or damage immunogens.

Chapter 5

5. Detection of lipolytic activity in mycoplasmas

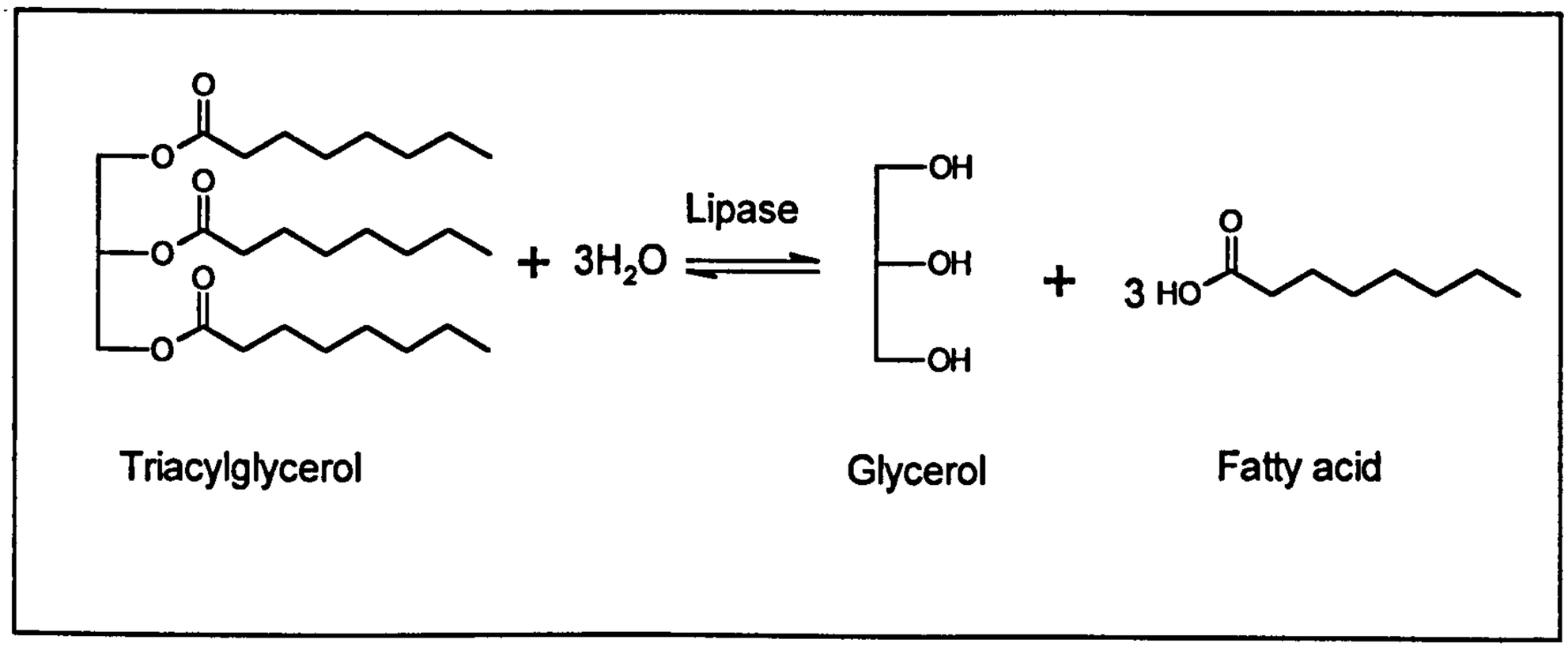
5.1. Introduction

Lipase (triacylglycerol hydrolases EC 3.1.1.3) is the trivial name given to enzymes that hydrolyse ester bonds of triacylglycerols to yield free fatty acids, diacylglycerols, monoacylglycerols, or carry out esterification of acids, alcohols and interesterification (Figure 5.1). These enzymes belong to the family of serine esterases and are widely distributed throughout animals, plants and microorganisms. The role of lipases in the physiology and the pathogenicity of mycoplasmas is yet to be fully elucidated. Sztager *et al.* (1988) and Ionta *et al.* (1997) considered microbial lipases to have great potential for commercial application due to their stability, selectivity and substrate specificity. Lipases from bacteria, e.g. various species of *Pseudomonas*, have also proved to be useful both in organic reactions and in the detergent industry. Many of them have been purified, characterised and their coding genes cloned (Jaeger *et al.*, 1996). Currently most lipases produced commercially are obtained from fungi and yeast (Jaeger *et al.*, 1994) however, interest in bacterial lipases has increased as they are more stable than those from other organisms, especially when exposed to high temperatures and other severe conditions. Moreover, enzymes from thermophilic bacteria have even higher stability under severe operational and storage conditions (Sugihara *et al.*, 1992). Most microorganisms produce extracellular lipase under suitable conditions but few have been studied. The organisms which possess substantial lipase activity are referred to as lipolytic and are associated with the spoilage of dairy products (Shelley *et al.*, 1987). The importance of microbial lipases results from their significant role in bacterial lipid metabolism, their involvement in pathogenic processes, and their use in biotechnology (Jaeger *et al.*, 1999).

Some lipolytic enzymes also hydrolyse water-soluble monomeric substrates with short chain fatty acids, such as tributyrin and some of them show no interfacial activation, thus having typical features of esterases. Uncertainty exists whether these enzymes should be classified as lipases or esterases. Lipolytic enzymes from *Pseudomonas aeruginosa* and *Bacillus subtilis*, react with monomeric substrates as well as with the emulsions, show no interfacial activation (Jaeger *et al.*, 1994). True lipases are dependent on the lipid-water interface in substrate emulsions to obtain their full activity, a phenomenon which has been described as interfacial activation (Sarda and

Desnuelle, 1958). Lipases are secreted in the pro-lipase form and the processing of the pro-lipase form to the mature enzyme occurs extracellularly by a specific protease. Interestingly the pro-lipase reveals little activity compared to the mature lipase (Gotz *et al.*, 1998).

Figure 5.1 The catalytic action of lipases. A triglyceride can be hydrolysed to form glycerol and fatty acids, or the reverse (synthesis) reaction can combine glycerol and fatty acids to form the triglyceride.



Mycoplasmas are nutritionally fastidious bacteria, which grow relatively slowly giving only low yields. Incubation periods are generally 2-5 days or longer. Even so in broth cultures, visible turbidity is not achieved, and on agar plates, colonies cannot be seen without the aid of a microscope. Selective media enabling only the growth of mycoplasmas have been formulated by the addition of antibiotics (e.g. ampicillin) acting against cell-walled bacteria. Mycoplasmas completely lack cell walls and are therefore totally resistant to ampicillin even at low concentrations. Following isolation, *Mycoplasma* species identification is usually based on serological tests, however in most cases, isolates are screened for their reaction in biochemical tests prior to serological analysis, to reduce the number of target species. The most widely used serological tests are also usually based on growth, the ability of antiserum in paper discs to cause zones of growth inhibition on agar media. Consequently, the routine identification of isolates may take several days after the first demonstration of their presence by their growth on agar plates. The detection of lipolytic activity is of significance in the identification of these isolates and certain other pathogenic organisms. Lipase activity is often difficult to distinguish from esterase activity. A variety of techniques have been used to detect lipolytic activity including: titrimetric

and indicator dye methods for detection of free fatty acids which released from the hydrolysis of triglycerides with the production of insoluble free fatty acids from solubilised lipids (Tweens 20 and 80); enzyme immunosorbant assay of lipases; and the use of chromogenic and fluorogenic substrates. Previously used chromogenic substrates, such as indoxyl and *p*-nitrophenyl esters, are water soluble, not generally considered to be true lipase substrates, and may also be hydrolysed by esterases (Shelley *et al.*, 1987). Recently, *Salmonella* agars incorporating chromogenic substrates have become commercially available. Poupart *et al.* (1991) used a chromogenic β-galactosidase substrate together with glucuronate, which is metabolised by salmonellae. Cardenas *et al.* (2001) have used chromogenic esters for detection of lipolytic activity in soil bacteria. Xylem peroxidase was assayed histochemically using the chromogenic substrate TMB (Ros Barcelo, 1998). Sugihara *et al.* (1992) and Kojima *et al.* (1994) described a standard methodology for the detection of lipolytic activity from lipase-producing microorganisms by using solid media supplemented with emulsified triglycerides. Esters of 4-methylumbelliferone have previously been used to screen for microbial esterase activity. Pancholy and Lynd (1971) used 7-butanoyloxy-4-methylumbelliferone to detect esterase-positive soil bacteria and fungi. Aguirre *et al.* (1990) used a C_8-esterase spot test in which a solution of methylumbelliferyl caprylate in ethanol was applied to colonies on Rambach agar and the appearance of blue fluorescence under UV light within 1 min of application was observed for all salmonellae test strains, however colonies of some *Pseudomonas* and *Acetobacter* also fluoresced. Ester substrates have been used in the detection of *Salmonella* (Miles *et al.*, 1992; Cook *et al.*, 1999). Esterase hydrolyses short chain organic acids esters and lipases are present in all organisms to various extents. Their specificity to various esters chain length is also variable.

The ability of *Acholeplasma* and a range of *Mycoplasma* species to hydrolyse esters of SLPA was determined in the present study. Chromogenic esters were used as substrates for the detection and preliminary characterisation of lipase activity in mycoplasmas. Esters differing in chain length (C_2-C_{10}) were first used to assess the specificity. The hydrolytic activity of lipases can be determined by numerous methods. They usually examine the products of the reaction: glycerol measured by a UV-method which is coupled with irreversible enzyme reactions or a volumetric method (Wahlefeld, 1974). Fatty acid production is monitored by HPLC, gas chromatography,

thin layer chromatography (Sugihara *et al.*, 1991), spectrophotometry or titrimetic method (Tietz and Fiereck, 1966).

The aim of this work was firstly the detection of lipolytic activity in mycoplasmas because there is very little information available concerning lipase-producing mycoplasmas. Secondly, a rapid biochemical test was developed using novel chromogenic substrates, which might be used for preliminary testing of mycoplasmas. An attempt was also made to detect lipase activity on SDS-PAGE for the determination of molecular weight. Finally, the detection of lipases may be important as a method of preliminary identification of highly pathogenic mycoplasmas.

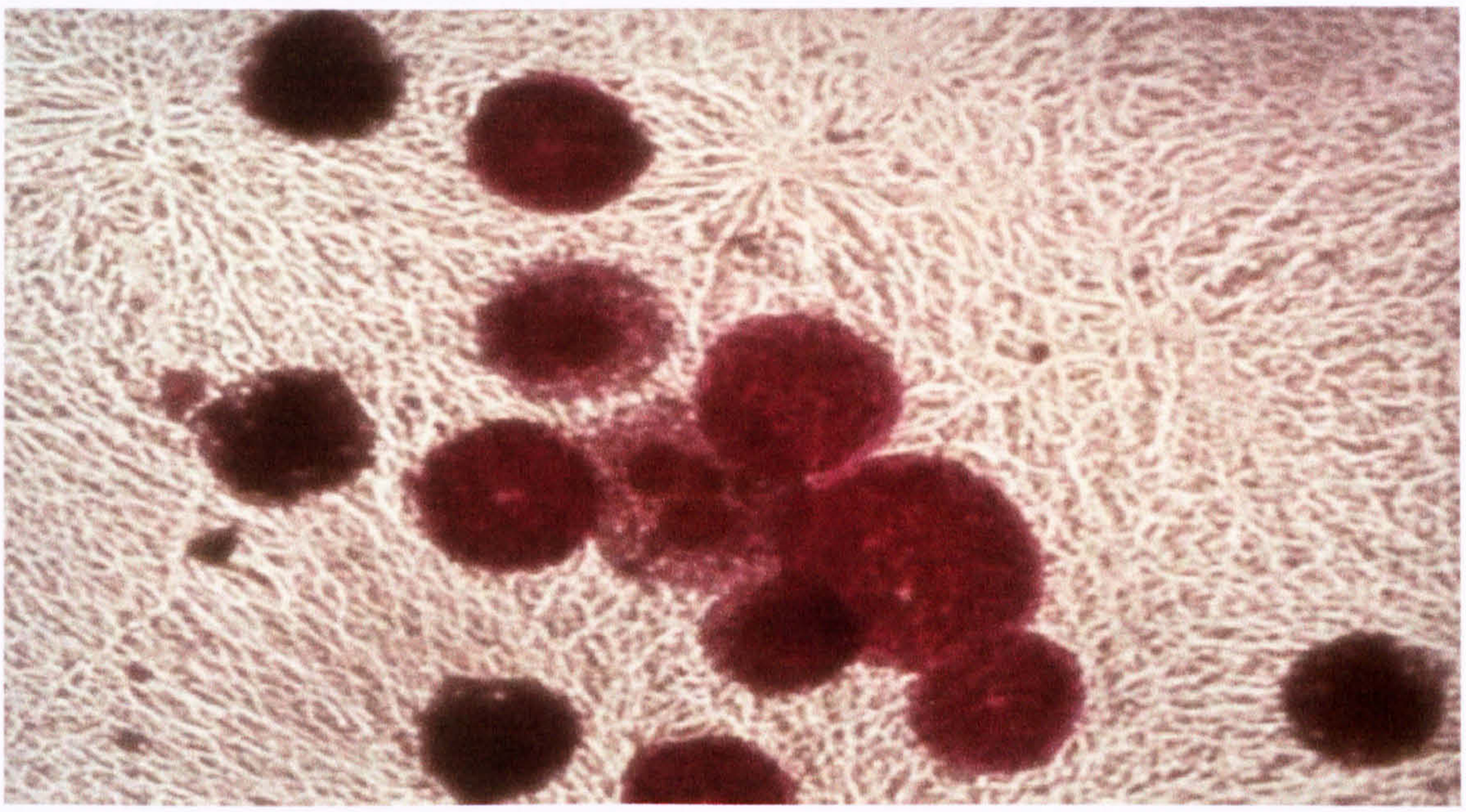
5.2 Results and discussion

5.2.1Development and optimisation of a chromogenic detection system for mycoplasmas

The conditions for qualitative lipase assay were optimised. A chromogenic C_8 organic acid ester known as 4-[2-(4-octanoyloxy-3,5-dimethoxyphenyl)-vinyl]-quinolinium-1-(propan-3-yl carboxylic acid) bromide (SLPA-octanoate; bromide form) was used for the detection of lipolytic activity in mycoplasmas. It was used at various concentrations and it was found that 1.25 mg/ml of SLPA-octanoate was optimal. The colonies were bright red and the reaction was fast. The optimal volume of the substrate, determined by using different volumes in the range 200-1200 µl, was found to be 400 µl, which was sufficient to cover the 60 mm blood agar plate. It is recommended that solutions of the substrate should cover the whole plate and the colonies.

The effect of chromogenic substrates on the colonies was also determined. It was found that the substrate did not cause any adverse effect on the viability of the colonies. Lipase-positive and lipase-negative mycoplasma colonies were grown on blood agar and were then subjected to the substrate dissolved in Ringer-HEPES buffer. After the reaction, single colonies were picked off and were grown in the broth medium. After growth in broth, they were again grown on blood agar plates, the colonies again subjected to the SLPA-octanoate and the same results were obtained. This was a very significant because this method can be used for their preliminary identification. Milk samples were directly cultured on the blood agar plates and after the growth of the mycoplasmas, colonies were subjected to SLPA-octanoate. Positive results were obtained with the appearance of bright red colonies within an hour (Figure 5.2).

Figure 5.2 Hydrolysis of SLPA-octanoate by colonies of *M. agalactiae*. Plates were inoculated with milk from an infected sheep.



5.2.1.1 Importance of colony age

The age of the colonies had a significant effect on lipase activity. It was observed that as the colonies became older, the rate of hydrolysis was slow. *M. bovis* was inoculated on agar plates and colonies were tested after 24, 48, 72, 96, 120, 144, 168 and 192 hours for SLPA-octanoate hydrolysis. The optimum age of the colony for the assay was determined as 24-72 hours. The age of the colonies affected the reaction and had a drastic effect upon hydrolysis. Colonies from 96-168 hours old showed a very delayed reaction but surprisingly, the 192 hours colonies showed reaction very similar (+ve reaction <1h) to that of 24-72 hours old colonies (Table 5.4). This was also significant because when colonies were viable, the substrate was transported into the cell and subsequently rapid hydrolysis occurred. When the colonies were old and not very viable, the reaction was very slow. In contrast the fast reaction of colonies at 192 hours showed the colonies had lysed and the enzyme released into the medium. This might caused the rapid hydrolysis of the SLPA-octanoate substrate. This result showed the possible intracellular location of the lipase enzyme.

5.2.1.2 Substrate specificity

In order to determine substrate specificity, chromogenic substrates of different chain lengths (C_2-C_{10}) were tested (Figure 2.2). It was shown that only SLPA-octanoate gave a positive reaction within an hour for *M. bovis* and *M. agalactiae.* SLPA-decanoate and SLPA-hexanoate were hydrolysed, more slowly (>1h) while acetate, propionate and butyrate were negative (Table 5.3). These results were in line with Lesuisse *et al.* (1993) who found *Bacillus subtilis* lipase enzyme has a preference for the 1,3 position and prefers fatty acids with a chain length of eight carbons. There is a great biotechnological interest in this *B. subtilis* enzyme because of its remarkable alkaline stability.

5.2.1.3 Solubility of SLPA-esters

The solubility of the SLPA-esters was also determined in different solvents and was tested on mycoplasma colonies on agar plates. The solvents used in the study were dimethyl sulphoxide (DMSO), methanol, and 2-methoxyethanol. SLPA-octanoate was dissolved in five different concentrations of these solvents: 10, 20, 30, 40, and 50 % (v/v) in Ringer-HEPES buffer. The substrate (12.5mg) dissolved in 10 ml of methanol and 2-methoxyethanol in Ringer-HEPES and was mixed by stirring. The solutions of

the SLPA-octanoate were flooded on the colonies and were incubated at 37°C. Methanol and 2-methoxyethanol, at 10, 20, 30, 40 % in each case gave a positive reaction in two hours, but the substrate had precipitated to some extent. However, 50 % methanol and 50 % 2-methoxyethanol gave a clear substrate solution and reaction was positive within 30 minutes in both the cases. The reaction was very slow for DMSO and was less satisfactory as the substrate was not dissolved completely leaving a cloudy solution. SLPA-octanoate (1.25 mg) was dissolved in 1 ml of the methanol and was evaporated overnight. At the time of use the substrate was reconstituted by adding either Ringer-HEPES or distilled water and the reaction was positive within 30 mins.

The substrate (12.5 mg) dissolved in 1 ml methanol, evaporated and reconstituted by adding 1ml of the Ringer-HEPES but in this case the substrate was not dissolved completely and rather sedimented. It did not cover the whole colonies, the reaction was very slow and colonies were partially red. Dannert *et al.* (1994) have reported that lipase activity was stable in the presence of methanol. So it is confirmed from experiments that methanol 50 % (v/v) is good solvent for SLPA-octanoate substrate.

5.2.1.4 Effect of addition of BSA

Bovine serum albumin (BSA), which is considered to enhance enzymatic activity, was used in combination with substrate in different concentrations (0, 2, 4 and 5 %; w/v). It did not enhance the lipolytic activity and reaction was unchanged.

5.2.1.5 Effect of temperature on plate assay

Plates were incubated at different temperatures, 4°C, 37°C, 42°C and room temperature (18°C) for the determination of optimum temperature. The optimum temperature was 37°C and the reaction at this temperature was complete within 30 mins; while incubation at 42°C showed partial hydrolysis, and that at 18°C and 4°C showed no hydrolysis even after 24h incubation. Plates kept at 4°C and then incubated for 30 mins at 37°C showed positive reaction within an hour.

5.2.1.6 Stability of substrate on storage

SLPA-octanoate substrate was stored at 4°C for 2, 7, and 14 days prior to testing. The reaction in all cases was positive within an hour. The substrate should be warmed at 37°C prior to use. SLPA-substrate also stored at –20°C for 14 days then thawed and incubated at 37°C for 30 mins gave the same reaction as that of the freshly prepared substrate showing that substrate storage did not affect its stability.

5.2.1.7 Effect of different animal sera and freeze-drying

Sera from different animals e.g. bovine calf, horse and porcine sera were used in blood agar base and all sera showed the same results (colonies of *M. agalactiae* and *M. bovis* were bright red within an hour). The SLPA-octanoate was freeze-dried for convenience and field use: 50 mg of substrate was dissolved in 40 ml of Ringer-HEPES with 1 % (v/v) Tween-20 and 1% (w/v) lactose. Aliquots of the substrate (1ml) were dispensed in bottles and freeze-dried overnight, and then kept at 4°C. The substrate was reconstituted by adding 1ml distilled water and used on the mycoplasma colonies along with a control. It was observed several times that freeze-dried substrate was not hydrolysed by the mycoplasma colonies. Subsequently it was found that the detergents adversely affected the lipase activity (Section 5.3.4).

5.2.2 Qualitative lipase assay method

Lipase activity was determined using novel chromogenic substrates. SLPA-ester solutions (400 µl) were applied to colonies and plates were incubated at 37°C for the development of colour. The mycoplasma colonies were observed under the microscope every 15 mins. Ester hydrolysis within limited time (< 1h) was indicated by the appearance of a bright red colour and was recorded as positive.

5.2.3 Detection of lipolytic activity using chromogenic substrates

The ability of *Acholeplasma* and a range of *Mycoplasma* species (Table 5.1) to hydrolyse fatty acids esters comprising SLPA-acetate, SLPA-propionate, SLPA-butyrate, SLPA-hexanoate, SLPA-octanoate and SLPA-decanoate (Section 2.20, Figure 2.2) was determined. After colony growth (24-72 h), plate surfaces (60 mm) were flooded with solutions of each ester in Ringer-HEPES buffer (400 µl), incubated at 37°C and the plates examined for the appearance of coloured colonies indicating

hydrolysis of the substrates. The development of red colonies was observed using a plate microscope (x 20 magnification). There was no colour change with short chain fatty acid ester (Table 5.3). All test species were tested for SLPA-hexanoate and SLPA-decanoate, and all strains, which were negative for SLPA-octanoate, were also negative for SLPA-hexanoate and SLPA-decanoate (Table 5.2).

The colonies of *M. agalactiae* and *M. bovis* became bright red after application of octanoate and decanoate esters: colour was evident within 30-60 mins with octanoate, 1-2 h for decanoate and 2-3 h for hexanoate. In contrast, strains of all other test species gave no colour reaction after 24-hour incubation. *M. verecundum* and *M. canis* colonies were not stained red, but a diffuse red area around colonies was visible after 3 hours and overnight incubation respectively (Table 5.1). The biochemical features of *M. verecundum,* which have been identified, are possession of phosphatase activity and ability to form 'film and spots' on agar (Holt *et al.*, 1993).

All organisms presumably possess some lipase/esterase activity. Lipolytic activity in a number of *Mycoplasma* species, including *M. agalactiae* and *M. bovis*, is indicated by their ability to form "film and spot" reactions on agar. The ability to hydrolyse SLPA-octanoate rapidly is not equivalent to the "film and spot" reaction since a number of "film and spot" positive species did not hydrolyse the SLPA esters (Table 5.1). It is possible that in *M. agalactiae* and *M. bovis,* lipase/esterase activity is high compared to that in other "film and spot" positive mycoplasmas. Alternatively, the difference in reaction may be due to the specificity of the enzyme. *Salmonella* appears to possess esterase, which has highest specificity for C_8 esters (Cook *et al.*, 1999).

The ability to lyse SLPA-octanoate may be useful in the rapid identification of *M. agalactiae* and *M. bovis*. *M. agalactiae* and *M. bovis* possess C_8 esterase activity, which may be detected rapidly using the chromogenic substrate SLPA-octanoate and this is a property associated only with these mycoplasmas. It is not a general feature of "film and spot" positive cultures. Also, *M. agalactiae* and *M. bovis* strains themselves may or may not be "film and spot" positive. These two species showed positive reaction because they are phenotypically very similar and were originally classified within the same species. Cross amplification of *M. agalactiae* and *M. bovis* in the heterologous PCR system showed that these two species are closely related and have

only eight nucleotide differences in the 16S rRNA sequence which corresponds to 99.5% nucleotide similarity (Yleana *et al.*, 1995).

These results were consistent with those of many workers who also used chromogenic substrates for the detection of bacteria. Chromogenic substrates have been used for the determination of peptidases in *Mycobacteria* (Muftic, 1967). Bashiruddin and Windsor (1998) developed a medium in which *M. mycoides* colonies were coloured red due to tetrazolium reduction. Using clinical material to inoculate plates, *M. mycoides* LC and *M. capri* colonies were dark red in 3 days while *M. mycoides* SC took seven days to become dark red, whereas using the SLPA-octanoate substrate on *M. agalactiae* and *M. bovis* colonies, reaction was very quick (bright red <1h). Recently, Rice *et al.* (2000) have developed a test based on hydrolysis of the chromogenic substrate α-glucosidase (maltase) (p-nitrophenyl-α-D-glucopyranoside α-glcosidase, pNPG, colourless), to give brightly coloured product (p-nitrophenol, yellow). The chromogenic substrate 3-diaminobenzidine (DAB) has also been used for the detection of H_2O_2 by mycoplasmas (Rice *et al.*, 2001).

These simple and rapid procedures used to detect lipolytic activity may also be used in other microorganisms. Stead (1983) suggested that most microorganisms possess some esterase and lipase activity. Qualitative assay will be important in distinguishing organisms possessing high levels of activity e.g. in food spoilage, or of value in identification procedures. The lipase enzyme is a known virulence factor in some bacteria and lipase production in *Pasteurella multocida* may be a virulence factor of disease (Joel *et al.*, 1999). The possession of high esterase/lipase activity in *M. agalactiae* and *M. bovis* may be of significance in their pathogenicity, resulting in the destruction of host cells.

5.2.4 Development of the field kit

The field kit was developed by dissolving 12.5 mg of the SLPA-octanoate in 10 ml of the 50 % (v/v) methanol in Ringer-HEPES (pH 7.6) sufficient for 25 x 60 mm plates. The solution of SLPA-substrate (400 µl) was flooded on the 24-72 hours old colonies and incubated at 37°C. Colonies were observed under the microscope for the appearance of bright red colour.

The substrate may also be dried and reconstituted at the time of use i.e. 1.25 mg of the SLPA-octanoate in 1ml of the methanol and evaporated and kept the dried substrate at 4°C and at the time of use added 1ml of the Ringer-HEPES pH 7.6. The test can be carried out on washed cells resuspended in Ringer- HEPES buffer. The development of blue colour is the indication of SLPA-hydrolysis, which gives a bright blue coloured SLPA-phenol (reaction mixture Section 5.3.2).

5.2.5 Effect of CO_2 on lipase activity

M. bovis was grown on blood agar and octanoate substrate (400 µl) was flooded on the colonies and then incubated in the anaerobic jar with CO_2 and catalase. Controls were also run on colonies aerobically in a normal incubator at 37°C. Under aerobic conditions there was no reaction at all and the pH of the agar was lowered to less than 7.0 in the presence of 10 % CO_2 in air. Control plates were positive within 30 mins and the pH of the plates was 7.6. These experiments were repeated several times and same results were obtained each time.

Results showed that the low pH of the medium affected the reaction. To investigate this, the cytoplasmic fraction of *M. bovis* cells along with SLPA-octanoate was bubbled with CO_2, and lipase activity and pH of the reaction were measured. It was shown that when the pH of the reaction mixture was lowered lipase activity was very low compared to the control. The chromogen (SLPA-phenol) was dissolved in the Ringer-HEPES and subjected to CO_2; the pH (7.6) and colour of the solution was recorded. The results showed that, as the bubbling progressed the colour and the pH of the mixture changed. The colour of the chromogen was dark green or dark blue and the pH was alkaline before bubbling. The colour was changed to yellow as the pH lowered (pH 6.5).

SLPA-octanoate substrate was dissolved in different pH PBS buffer and it was shown that in acidic buffers, the substrate was not dissolved but it precipitated. When the pH of the buffer was increased to neutral and alkaline levels the substrate dissolved completely. This can be readily understood by the fact that the acid carboxyl group is ionized at higher pH and therefore becomes more soluble. Therefore it was shown that to obtain high rates of SLPA-octanoate hydrolysis, the pH must be greater than 7 and optimally 7.6.

Table 5.1 Hydrolysis of SLPA-octanoate by *Mycoplasma bovis* species

Species	Strains tested	Film and spot reaction	Development of colour
M. agalactiae	26	usually positive	< 1hour
M. bovis	27	usually positive	< 1hour
M. bovirhinis	5	negative or positive	negative
M. alkalescens	1	negative	negative
M. canis	12	negative	negative*
M. columbinum	1	positive	negative
M. caviae	1	positive	negative
M. columbinasale	1	positive	negative
M. edwardii	1	positive	negative
M. dispar	3	negative	negative
M. felis	1	positive	negative
M. fermentans	7	positive	negative
M. gallinarum	1	positive	negative
M. immitans	1	negative	negative
M. mycoides	15	negative	negative
M. penetrans	1	negative	negative
M. putrefaciens	1	positive	negative
M. salivarium	1	positive	negative
M. verecundum	1	positive	negative*
M. ovine serogroup 11	3	negative	negative
A. laidlawii	1	negative	negative
A. oculi	1	negative	negative
A. axanthum	1	negative	negative

* Diffuse red colouration around colonies after ≥ 3 h

Table 5.2 Hydrolysis of SLPA-hexanoate and SLPA-decanoate by *Mycoplasma s*pecies

Species	**Strains tested**	**Devlopment of colour**	
		Hexanoate	**Decanoate**
M. alkalescens	1	negative	negative
M. bovirhinis	7	negative	negative
M. canis	12	negative	negative
M. columbinum	1	negative	negative
M. caviae	1	negative	negative
M. columbinasale	1	negative	negative
M. edwardii	1	negative	negative
M. dispar	3	negative	negative
M. felis	1	negative	negative
M. fermentans	6	negative	negative
M. gallinarum	1	negative	negative
M. immitans	1	negative	negative
M. mycoides	15	negative	negative
M. penetrans	1	negative	negative
M. putrefaciens	1	negative	negative
M. salivarium	1	negative	negative
M. verecundum	1	negative	negative
M. ovine serogroup 11	3	negative	negative
A. laidlawii	1	negative	negative
A. oculi	1	negative	negative
A. œxanthum	1	negative	negative
M. agalactiae	26	positive	positive
M. bovis	27	positive	positive

The colonies of these *Mycoplasma* species were incubated at 37°C for more than five hours continuously. There was no development of colour, however control experiment with *M. bovis* and *M. agalactiae* showed positive reaction. The colonies were also incubated overnight but there was no hydrolysis.

Richardson and Cook (1998) reported that during the development of diagnostic tests using chromogenic substrates it was found that for organisms possessing lipase activity, colour intensity was modified by pH. The chromophore of SLPA-octanoate becomes protonated at lowered pH values and the protonated form is only weakly coloured.

5.2.6 Confirmation of lipolytic activity by pH change using glycerol tributyrate substrate

On hydrolysis of glycerol tributyrate, fatty acids are produced which lowered the pH, which can be monitored on the pH meter. The aim of this experiment was to confirm the presence of lipolytic activity. *M. bovis* metabolised the highest concentration of glycerol tributyrate and pH was determined as in Section 2.12. It was shown that glycerol tributyrate (10 % w/v) was metabolised and subsequently the pH of the reaction was lowered from 7.6 to 6.4. It showed that lipase caused the hydrolysis of the substrate and liberated fatty acids, which caused a decrease in pH. The enzyme may be denatured by the high acidity developed. This result also confirmed the presence of lipase activity in *M. bovis*. Glycerol tributyrate has been used for the identification of *Moraxella catarrhalis* (Perez *et al.*, 1990) and was a useful and rapid test. So glycerol tributyrate can also be used for the detection of lipolytic activity in mycoplasmas.

5.3 Quantitative detection of lipolytic activity in *Mycoplasma bovis*

5.3.1 Comparision of spectrophotometric and fluorimetric lipase assays

5.3.2 Development of spectrophotometric lipase assay

A lipase assay was developed using SLPA-octanoate chromogenic substrate. The conditions were optimised for the assay. The reaction mixture consisted of 100 µl of either cytoplasmic fraction or membrane fraction or whole cells, in 900 µl of Ringer-HEPES buffer and 50 µl of 1 mM chromogenic susbtrate. The reaction mixture for the assay was optimised using different volumes of the cytoplasmic, membrane fractions of *M. bovis* and different concentrations of SLPA-octanoate substrate.

The substrate for the spectrophotometric assay was dissolved in buffer A which contained (g/l): boric acid, 3.09; potassium chloride, 3.75; and deoxycholic acid (sodium salt), 14.4; the pH was adjusted to 7.6 with 0.1 M sodium hydroxide. The

substrate is a pale yellow solution. The enzyme assay was conducted at 37°C and incubation times were between 30 mins to 2 hours. Reactions were stopped by adding 335 µl of a solution of 1,4-diazobicyclo (2.2.2) octane (112.2 g/l in 80 % (v/v) acetone. Absorbance was measured at 595 nm and phenol released was determined with reference to standard curve (Appendix 2).

5.3.3 Spectrophotometric lipase assay

A rapid and convenient spectrophotometric lipase assay was developed and conditions were optimised (Section 5.3.2.). The absorption spectrum for the SLPA-phenol, (λ_{max}) was determined at 595 nm, which gave maximum absorption (Appendix 2). The optimal temperature for the lipase assay was determined as 37°C.

Lipase activity was determined at various temperatures (20-80°C). The rate of hydrolysis was reduced significantly at room temperature (20°C) and at 50°C (Figure 5.4). Experiments, also conducted at different pH values, showed that the optimum pH for the lipase assay was pH 7.5 in Ringer-HEPES and phosphate buffer (Na_2HPO_4. $2H_2O$ 15.0 g/l; NaH_2PO_4. $2H_2O$ 13.0 g/l) respectively (Figure 5.3). The SLPA-octanoate substrate was dissolved completely in phosphate buffer pH ranging from pH 7.0-8.0. The substrate was not dissolved completely at acidic pH and lipase activity was not detected at acidic pH. These results were in agreement with those of Oh *et al.* (1999) and Talon *et al.* (1995) who reported lipase as being unstable in acidic conditions and completely inactivated below pH 5.0. The activities were strongly decreased at acidic pH. In contrast, Rosenstein and Gotz (2000) found staphylococcal lipase stable under acidic conditions.

The optimal incubation times of reaction mixture were between 20 min to 2 hours depending on the lipase activity. The lipase activity was determined in whole cells, cytoplasmic and membrane fractions. The specific activity in the cytoplasmic fraction was 481 µmoles/min/mg cell protein while the specific activity in the membrane fraction was 408 µmoles/min/mg cell protein. The specific activity in whole cells of *M. bovis* of the same batch was also as 932 µmoles/min/mg cell protein (Table 5.5). Lipase activity was tested in lipase-negative species *M. mycoides* subsp. *mycoides* SC and no activity was shown in comparasion to *M. bovis*. These results suggested that the lipase activity in *M. bovis* was either intracellular or cytoplasmic.

5.3.4 Effect of detergents on lipase activity measured by spectrophotometric assay

The effect of different detergents on lipase activity was investigated since detergents are frequently added to bacterial media to increase selectivity. It was also considered likely that the detergent might aid the passage of chromogenic esters across the lipid membrane. Detergents are used to aid the freeze-drying of the substrate or in SDS-PAGE to determine the enzyme molecular weight. Different detergents such as SDS, Triton X-100, and Tween-20 were used in the study and their activity was measured as previously described (Section 5.3.2). All of these detergents affected lipolytic activity, causing a significant loss of activity, even at very low concentrations (Figure 5.5). These results were in agreement with those of Hiol *et al.* (1999) who found Triton X-100, Tween-20 and SDS strongly reduced lipase activity. Ransac *et al.* (1996) reported that surfactants might interfere with the catalytic function of lipases.

These amphiphilic molecules can inhibit lipase activity, and a correlation has also been found between the effect of surfactant and the extent of adsorption of lipase by the lipid interface. Patkar and Bjorkling (1994) have observed lipase inhibition by several organophosphorous compounds which inhibit lipase via irreversible phosphorylation of the serine residue at the active site. It has also been recognised that free fatty acids and alcohols inhibit lipase-catalysed hydrolysis reactions (Rostrup *et al.*, 1990). It is believed that this phenomenon is due to accumulation of these compounds at the lipid/water interface thereby blocking access of triglyceride molecules to the active sites. Plotkin *et al.* (1996) reported that the lipase activity of *Malassezia furfur* was inhibited by increasing concentrations of Tween-80. The results were also in line with Dannert *et al.* (1994) who reported that the addition of detergents such as Tween-20 and Tween-80 immediately decreased lipase activity

5.3.5 Effect of culture age on lipase activity measured by the spectrophotometric assay

M. bovis cells were grown for 7 days using single batch culture. Lipase activity was measured in whole cells and supernatant as previously described (Section 5.3.2) every 24 hour for 7 days. The highest activity was seen in the first 72 hours, and activity in the supernatant was very low. Between 96-120 hours the lipase activity was high in the supernatant, while in cells activity decreased.

The activity after 144 hours was very high in both whole cells and supernatant. It is likely the cells have lysed and caused the enzyme to be released outside the cell. These results were in agreement with previous results (Section 5.2.1.1) in which lipase activity was high in the 192 hours old plates (+ve <1h). These results suggested that the enzyme was either intracellular or cytoplasmic and also might be active in stationary phase. In *Candida rugosa* extracellular lipase normally appeared at the end of the exponential growth phase (Dalmau *et al.*, 2000).

5.3.6 Fluorimetric assay

The fluorimetric assay was conducted as previously described (Section 2.13.5). *M. bovis* whole cells, the cytoplasmic and the membrane fraction were assayed to determine location of lipase activity using the fluorimetric method. The esterase/lipase activity in the cytoplasmic fraction was 586 µmoles/min/mg cell protein, membrane fraction 414 µmoles/min/mg cell protein and activity in the whole cells was 808 µmoles/min/mg cell protein (Table 5.6). These results correlated with the spectrophotometric results and suggested that the enzyme was intracellular. The results obtained in the spectrophotometric and the fluorimetric assays showed more activity in the cytoplasmic fraction.

Using the fluorimetric assay lipase activity was higher in the cytoplasmic fraction compared to the spectrophotometric assay indicating that the fluorimetric technique is more sensitive. The intensity of fluorescence was correlated directly with the amount of 4-methylumbelliferone (MU) formed by using a standard curve of freshly prepared solutions (Appendix 2). Bacterial enzymes acting on 4-MU compounds have been used by Godsey *et al.* (1981) and Trepeta and Edberg (1984) in enzyme assays to identify faecal coliforms (Berg and Fiksdal, 1988) to measure exoenzymatic activity in natural waters. Fiksdal *et al.* (1989) used 4-methylumbelliferyl heptanoate to assay 4-methylumbelliferyl heptanoate hydrolase in *E. coli.* The fluorescence of 4-MU caused by the lipolytic hydrolysis of the acylated 4-MU is a measure of the activity of the lipase. The activity can be expressed either as the increase in fluorescent intensity per unit time or as the intensity of fluorescence correlated with the production of 4-MU catalysed by the lipase per unit time.

Table 5.3 Hydrolysis of SLPA esters by *M. agalactiae* and *M. bovis* colonies

SLPA-ester	**No of carbon**	**Hydrolysis by *M. agalactiae* and *M. bovis* strains**
Acetate	2	negative
Propionate	3	negative
Butyrate	4	negative
Hexanoate	6	2-3 hours
Octanoate	8	<1 hour
Decanoate	10	1-2 hour

Negative, development of red colour not seen after 24 h incubation

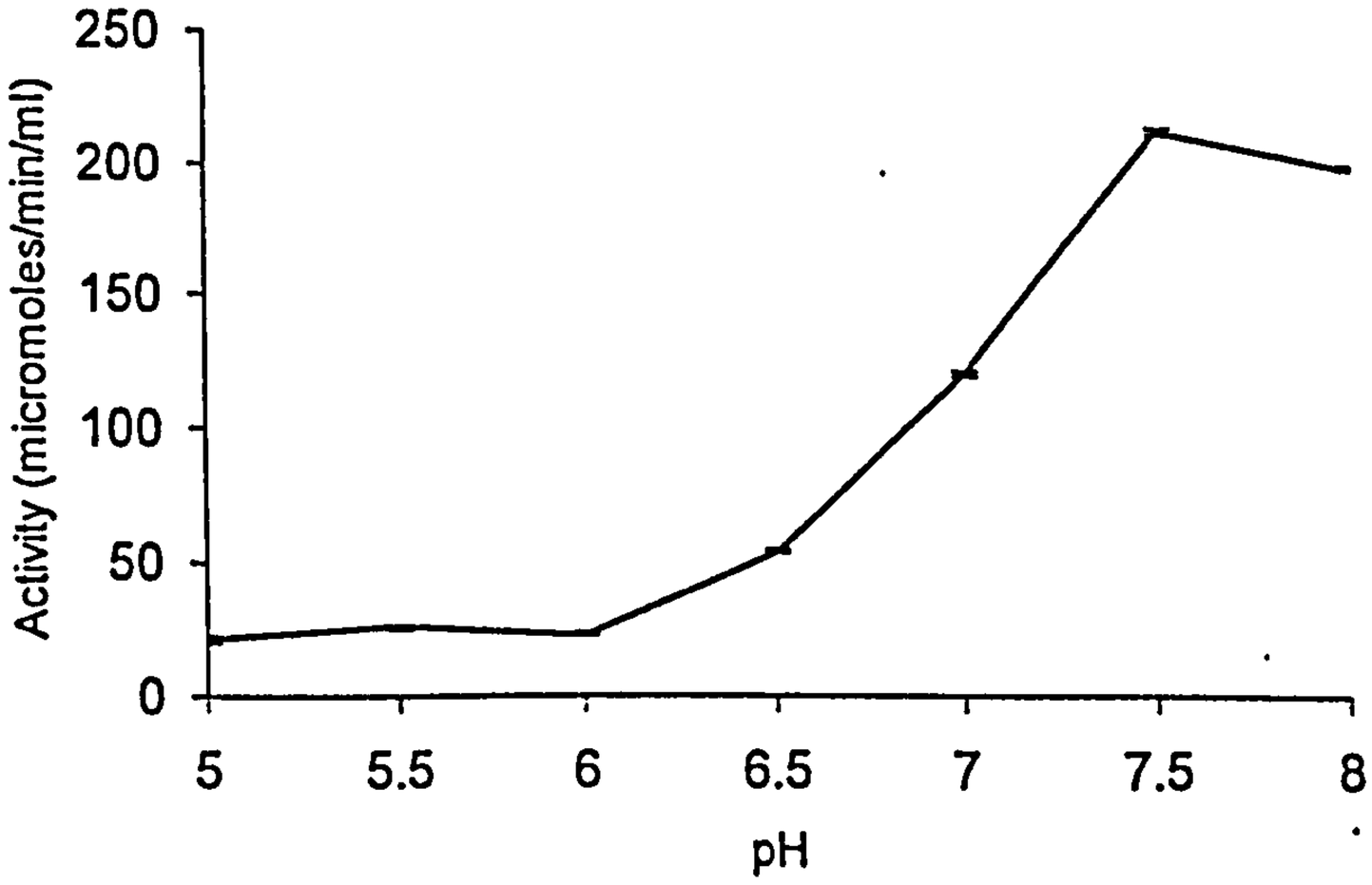
Table 5.4 The effect of age on the hydrolysis of SLPA-octanoate. The development of bright red colour indicated a positive reaction. Data of strains of *M. agalactiae* NCTC 10123, 453/93, 701/93, 101/94 and *M. bovis* NCTC 10131, 82B96, 193B96 and 135B99.

Name of species	Age of the colonies (hours)	Appearance of red colour				
		30–45 min	60-90 min	90-120 min	120-150 min	150-180 min
M. agalactiae	24	+				
	48	+				
	72	+				
	96	–	+			
	120	–	–	+		
	144	–	–	–	+	
	168	–	–	–	–	+
	192	+				
M. bovis	24	+				
	48	+				
	72	+				
	96	–	+			
	120	–	–	+		
	144	–	–	–	+	
	168	–	–	–	–	+
	192	+				

Figure 5.3 Effect of pH on lipase activity (micromoles/min/ml of whole cells) of *M. bovis* cells in phosphate buffer (A) and Ringer-HEPES (B).

Data are means ± SD of three independent experiments.

A



B

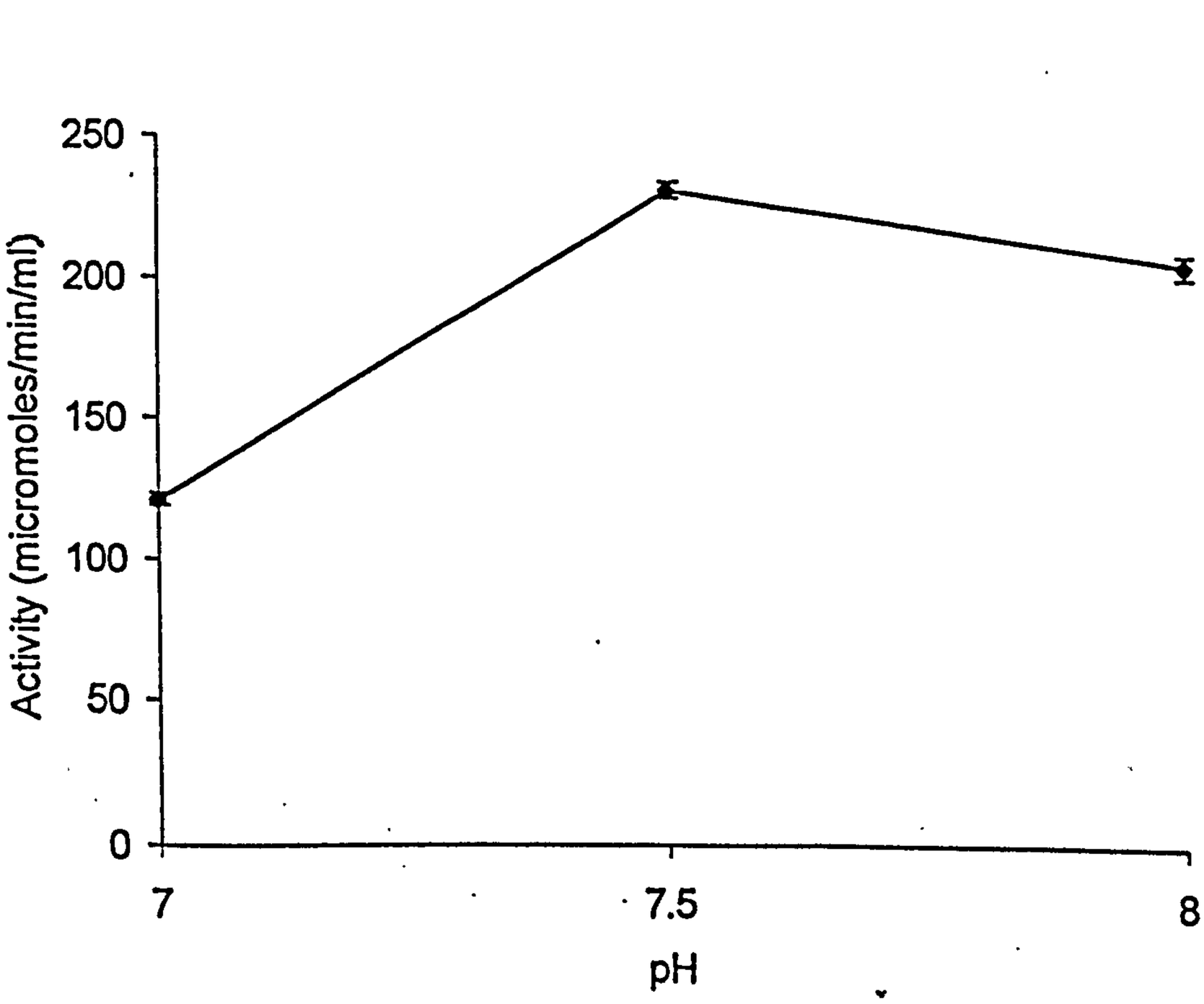


Figure 5.4 Effect of different temperatures on lipase activity (micromoles/min/ml of whole cells) in *M. bovis* cells. Data are means ± SD of three independent experiments.

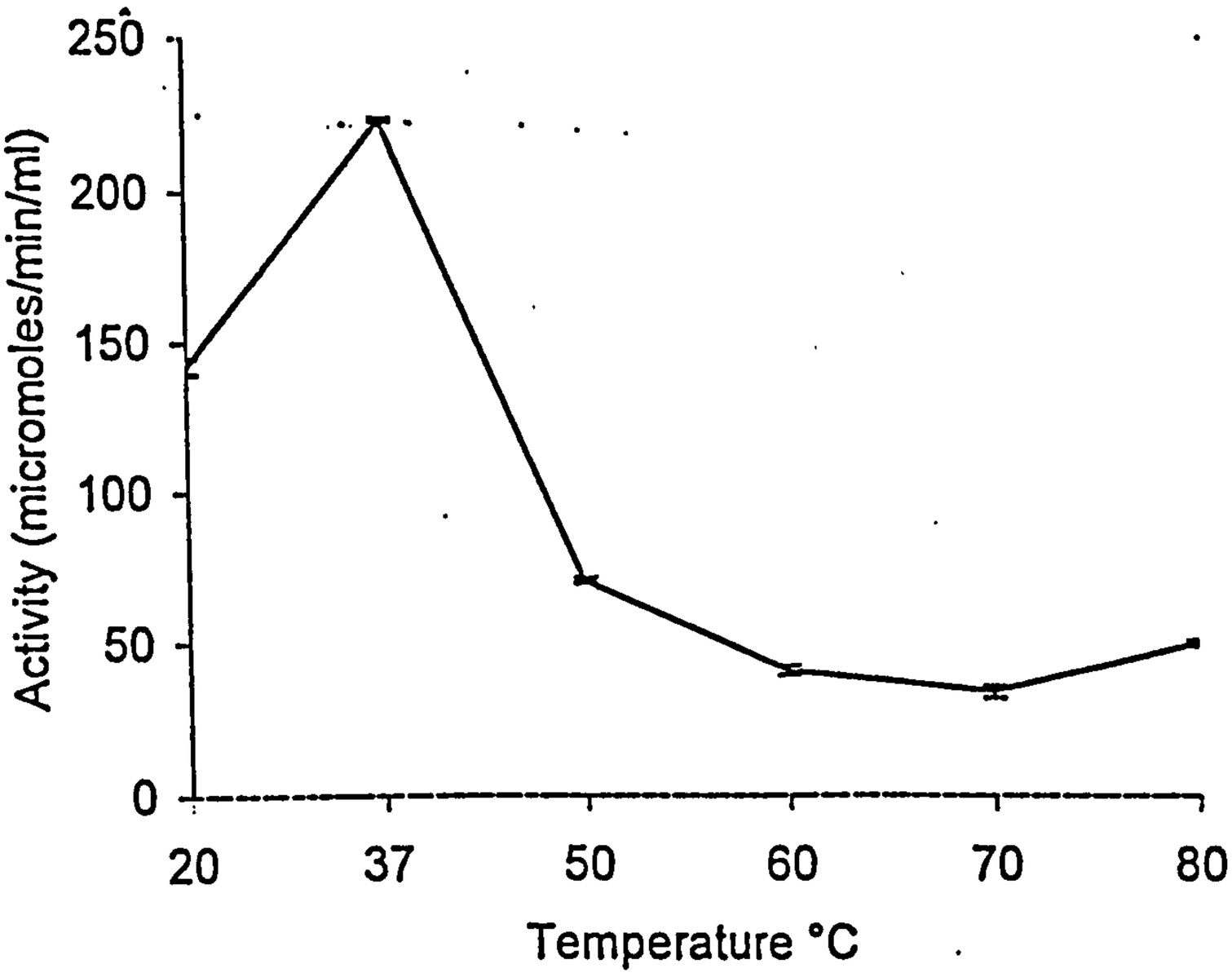
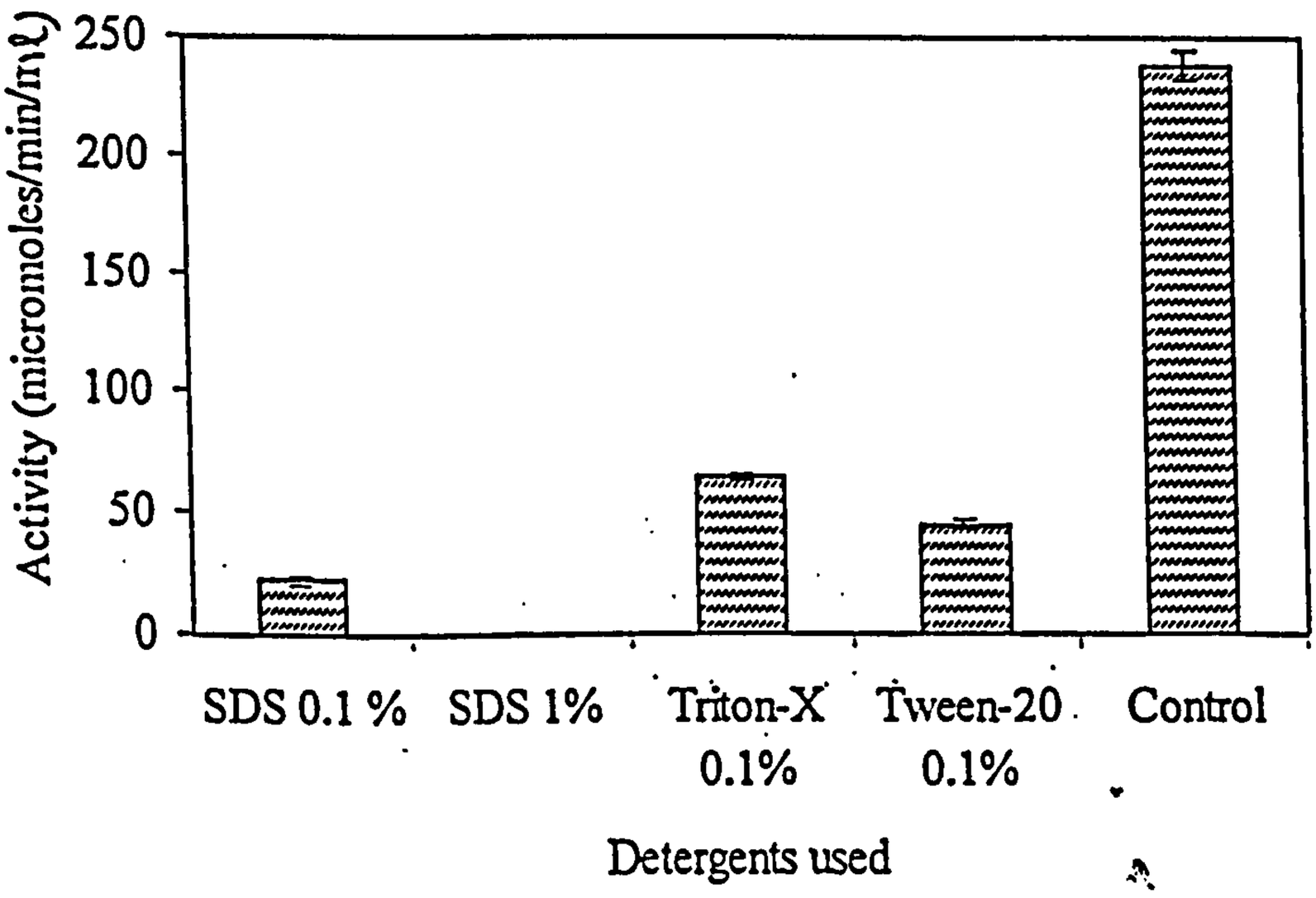


Figure 5.5 Effect of detergents on lipase activity (micromoles/min/ml of whole cells) in *M. bovis* cells. Data are means ± SD of three independent experiments.



Guilbaut *et al.* (1968) compared 12 different compounds as substrates for various lipases. They claimed that 4-MU heptanoate was the most suitable substrate for the fluorimetric assay of lipolytic activity. Deeth (1978) applied fluorimetric techniques in assaying lipases and esterases in milk using 4-methylumbelliferone heptanoate as the substrate. Gotz *et al.* (1998) showed that the activity of various *Staphylococus aureus* lipases is stimulated by divalent cations such as Ca^{++}. Gulomova *et al.* (1993) observed that Ca^{++} strongly enhanced lipase activity and Ca^{++} has been reported to form complexes with ionised fatty acids, changing their solubility and behaviour at interfaces. Garcia *et al.* (1991), Malcata *et al.* (1992) and Simons *et al.* (1997) reported the presence of Ca^{++} usually led to increased rate of hydrolysis and ester synthesis reaction rates. The activity was higher in the fluorimetric assay compared to the spectrophotometric assay which might be because of the effect of Ca^{++}. The fluorimetric method has been shown to be more sensitive than the titrimetric methods as a procedure for assay of lipase activity (Roy, 1979).

Perez *et al.* (1990) have described a rapid esterase test of value in the characterisation of *Moraxella, Neisseria* and *Acinetobacter* species. This test used 4-MU butyrate and a positive result was indicated by the development of a blue fluorescence on exposure to UV. The fluorimetric technique is very sensitive and requires a very small volume of cells. Lipolytic activity in all mycoplasmas can be detected by using this technique. The fluorimetric method used in this study was based on the hydrolysis of the fluorochrome 4-MU heptanoate by lipases of *M. bovis* cells. These methods are simple, reliable and reproducible. The fluorimetric technique has not been used previously for the detection of liploytic activity in mycoplasmas although it is used widely for the detection of lipases/esterases of other microorganisms.

5.3 Detection of lipolytic activity on polyacrylamide gels

5.4.1 Detection of lipase activity on SDS-PAGE

The gels were run as previously described (Section 2.14). *M. bovis* whole cells were analysed on SDS gels to determine the lipase molecular weight but lipase activity was not detected. Partial denaturing gel was used and the gels were renatured with Triton X-100, but no enzyme activity was detected. *Candida rugosa* (commercial enzyme) was also tested by SDS-PAGE but no enzyme activity was detected. SDS was used in

different concentrations (0.1-10% w/v) in gels and running buffer, but it was not possible to detect any esterase/lipase activity nor to establish the molecular mass of the lipase enzyme because of the effects of detergents, which inactivated the enzyme. These results suggested that the mycoplasma lipase enzyme is very sensitive to detergent.

These results were in line with Lincolin *et al.* (1994) who reported that acetylglucosaminidase activity was not detected by SDS-PAGE if the gel was left too long in the Triton X-100. This was the effect of detergent which inactivated the enzyme as previously described in Section 5.3.4. These results suggest that the mycoplasma lipase enzyme is very sensitive and denatured during the steps of SDS-PAGE. In contrast Rosenstein and Gotz (2000) determined the size of staphylococcal lipases by SDS-PAGE. Lipase from *Staphylococcus* was also separated by SDS-PAGE by Gotz *et al.* (1985) and Farrell *et al.* (1993). Dartois *et al.* (1992) found that *Bacillus subtilis* secretes a very small lipase with a molecular mass of 19.4-kDa.

5.4.2 Detection of lipase activity on native gels

M. bovis whole cells were run on native gels (Section 2.14). However, SDS was not added in sample buffer, gels and running buffer, and samples were not boiled. Lipase activity was detected for *M. bovis* and *Candida rugosa* commercial lipase, however no activity bands were detected for *M. putrefaciens* lipase-negative species. Two bands were seen for lipase activity using native gels for *M. bovis* and bands were seen for *Candida rugosa* (Figure 5.6). It is presumed these are dimers of the same enzyme and not isomers.

These results were in agreement with those of Lincolin *et al.* (1994) who found acetylglucosaminidase activity on polyacrylamide gels. Plotkin *et al.* (1996) were also able to detect lipase activity of *Malassezia furfur* on native gels. A methodology was developed for the detection of lipolytic activity in mycoplasmas on native gels. This technique was quick and easy to use providing a very sensitive method for the detection of mycoplasma lipase activity on polyacrylamide gels. The lipase enzyme was very sensitive, and activity was lost when SDS and Triton X-100 were used.

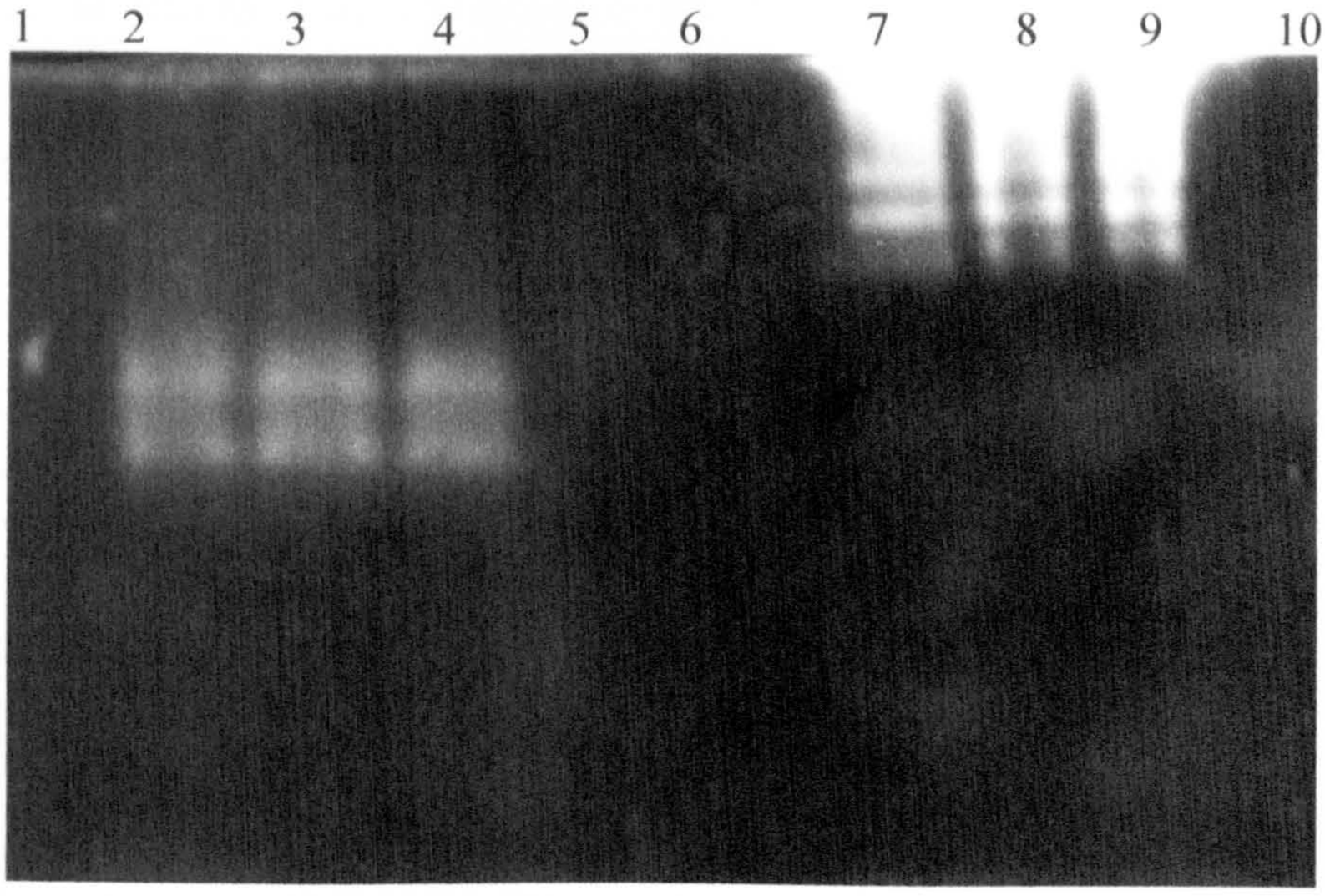
Table 5.5 Lipase activities in different fractions of *M. bovis* measured by spectrophotometric assay using 1 mM SLPA-octanoate.

Fraction	Total volume (ml)	Activity (μmoles/min/ total volume)	Total protein (mg)	Specific activity (μmoles/min/mg total protein)
Cytoplasmic	40	4481	9.3	481
Membrane	15	2190	5.4	408
Whole cells	1	233	0.250	932

Table 5.6 Lipase activities in different fractions of *M. bovis* measured by fluorimetric assay using 1 mM SLPA-octanoate.

Fraction	Total volume (ml)	Activity (μmoles/min/ total volume)	Total protein (mg)	Specific activity (μmoles/min/mg total protein)
Cytoplasmic	40	5460	9.3	586
Membrane	15	3165	5.4	414
Whole cells	1	202	0.250	808

Figure 5.6 Detection of lipase activity in *M. bovis* on native PAGE (12.5%). Lane 1, blank, lane 2, *M. bovis* NCTC 10131, lane 3 *M. bovis* NCTC 10131, lane 4 *M. bovis* NCTC 10131, lane 5 *M. putrefaciens*, lane 6 *M. putrefaciens,* lane 7 *Candida* lipase, lane 8 *Candida* lipase, lane 9 *Candida* lipase, lane 10 Blank



The substrate 4-MU heptanoate was most suitable for this technique. The results were in line with different workers, who also described lipase activity on native gels. The activity of copper-zinc superoxide dismutase in *M. hyopneumoniae* was detected on SDS and native polyacrylamide gels by Chen *et al.* (2000).

The lipase activity from thermophilic *Bacillus* sp was determined on native PAGE and the molecular mass was determined by SDS-PAGE. Hiol *et al.* (1999) detected lipase activity of *Mucor hiemalis f. hiemalis* on native gels and Gracia *et al.* (1997) detected lipolytic activity of filamentous fungi on native gels. Detection of lipolytic activity on native gels has a rapid method and has not been previously used for mycoplasmas. The method can be used for detection of lipolytic activity on native gels.

5.5 Conclusions

It was determined in this study that the ability to rapidly hydrolyse SLPA-octanoate was not equivalent to the film and spot reaction as a number of film and spot positive species were unable to hydrolyse SLPA-esters. *M. agalactiae* and *M. bovis*, which rapidly hydrolyse SLPA-esters, may have more lipolytic activity or the enzyme may be more specific for this substrate. The lipase assay demonstrated that the lipolytic activity was intracellular since more activity was seen in the cytoplasmic fraction. *M. agalactiae* and *M. bovis* possess C_8-esterase activity, which may be rapidly detected using the chromogenic substrate SLPA-octanoate.

The ability to lyse only the longer chain (C_6, C_8 and C_{10}) fatty acids esters of SLPA suggests that the activity detected is classical lipase activity rather than an esterase activity. This activity was absent in a range of other *Mycoplasma* species. Rapid hydrolysis of SLPA-octanoate might be useful in the presumptive identification of *M. agalactiae* and *M. bovis*. The possession of high esterase/lipase activity by these two species might be significant to their pathogenicity.

Lipolytic activity was inhibited by detergents and was enhanced by Ca^{++} ions. Lipase activity was not detected by SDS-PAGE and hence it was not possible to determine the molecular size of the lipase enzyme. However lipase enzyme activity was detected on native PAGE and shown to be dimer. In this chapter a simple rapid colorimetric procedure for the detection of *M. agalactiae* and *M. bovis* has been developed.

Chapter 6

6. Determination of relationship amongst type and field isolates of *Mycoplasma bovis* by restricted fragment length polymorphism (RFLP), restriction endonucleases analysis, pulsed field gel electrophoresis (PFGE), sodium dodecyl gel electrophoresis (SDS-PAGE) and immunoblotting.

6.1 Introduction

Mycoplasmal infections are usually diagnosed by serological procedures that are sometimes hampered by intraspecies cross-reactions and non-specific reactions. Moreover there are some *Mycoplasma* species showing antigenic heterogeneity and poor immune responses that makes the development of reliable serological procedures for detection more difficult (Jeffery *et al.*, 1995). In particular the confirmation of the identity of an isolate by growth inhibition requires considerable additional time, and is hampered by bacterial and fungal contamination. The principal disadvantage of growth inhibition methods in mycoplasma specification is the requirement for monospecific, high titred antisera. All species of the genus *Mycoplasma* have been classified on the basis of serological criteria, especially by use of tests such as growth inhibition and epi-immunofluorescence, because of their high specificity (Clyde, 1964; Rosendal and Black, 1972). However these tests may have failed to reveal taxonomic differences when applied to *M. bovis.*

There are numerous methods for comparing mycoplasmas; they are based mostly on total DNA digestion with frequently cutting restriction enzymes and analysis of the protein (Avakian *et al.*, 1991; Sachse *et al.*, 1992; Solsona *et al.*, 1996). Pyle and Finch. (1988) have used pulsed field gel electrophoresis (PFGE) for obtaining mycoplasma molecular fingerprints, which may be useful for epidemiological studies. In control of the diseases caused by *M. bovis* the main strategy is the strict isolation of infected animals from non-infected ones, which requires rapid, sensitive and reliable diagnostic techniques. Immunoblotting is used for the detection of specific antigens however detailed analysis of the antigen structure of *M. bovis* strains revealed that some antigens are stable, being present in all tested strains (Berthold *et al.*, 1992). At the same time, some of the antigens, like the antigenically and structurally related, variable surface lipoproteins VspA (63-65 kDa), VspB (46-kDa) and VspC (77-Kda)

(Rosengarten *et al.*, 1994) as well as pMB 67 (Behrens *et al.*, 1994) undergo high frequency variation in expression and size. Nevertheless it appears that these antigens participate in adhesion to the host cells (Sachse *et al.*, 1993). The extent to which *M. bovis* strains vary is not fully known however and a serodiagnostic assay based on a single antigen might not be sensitive to cover all *M. bovis* infections. The control of disease would be helped if a specific, sensitive and rapid diagnostic technique could be developed which can detect all *M. bovis* strains such as using monoclonal antibodies (Heller *et al.*, 1993) or nucleic acid probes (Mattsson *et al.*, 1991; McCully and Brock, 1992) and hybridisation with randomly cloned DNA fragment has been used for detection of *M. bovis*, but this system was found to cross hybridise with *M. arginini* (McCully and Brock, 1992). On the basis of biochemical characterisation (Chapter 3) it was found that all *M. bovis* strains tested for substrate utilisation exhibited no significant differences within the species nor did they differ biochemically from *M. agalactiae, M. bovigenitalium* and *M.* ovine serogroup 11.

The aims of this study were:

a) To investigate the genomic, protein and antigenic variability of UK *M. bovis* strains

b) To determine the protein variability of *M. agalactiae*, *M.* ovine serogroup 11 and *M. bovigenitalium* and to correlate these results with biochemical findings.

6.2 Results and Discussion

6.2.1 Analysis of *M. bovis* strains by PCR and restricted fragment length polymorphism (RFLP)

DNA was extracted (Section 2.15.1) and run on 0.7 % (w/v) agarose gel to check the purity of the DNA. All the strains showed a single band (Figure 6.1) and were confirmed by PCR using the oligonucleotide primer pair (Section 2.15.3) which amplify the 1.6 kb fragment from *M. bovis*, type strain NCTC 10131 and 15 field strains (Figure 6.2). The PCR product was digested by different restriction enzymes (Section 2.15.4). PCR product (5 µl) digestion by *Asn*I, showed identical restriction fragment profiles for all the strains (Figure 6.3) with four different bands of length 270 to 480 bp. Restriction enzyme *Ssp*1 also showed the same fragment patterns for all the strains (Figure 6.4). All amplified products showed identical restriction patterns with fragment sizes ranging from 300-650 bp. Restriction enzyme *Dde*I also showed identical

restriction pattern except for the highly passaged *M. bovis* strain 119B96 which showed two extra bands (Figure 6.5) of size 150-600 bp, and a very weak band (30 bp) was also shown for all the strains. The high passaged strain showed one major band lost when subjected to SDS-PAGE (Section 6.2.5). Evans *et al.* (2000) also found distinct differences in the DNA fragment pattern of passaged *M. gallisepticum* strains after digestion with restriction endonucleases. All the other strains showed the same fragment pattern. These results are very interesting as these passaged strains may have undergone a mutation and these mutants have also shown reduced H_2O_2 production (Chapter 4). The *uvr*C gene of *M. bovis* was determined to be 1761bp in length encoding proteins of 571 amino acids. The presence of the triplet UGA, a stop codon in most prokaryotes, is common within genes of mycoplasmas where it encodes the amino acid tryptophan. The *uvr*C gene of *M. bovis* contains one UGA_{Trp} codon which is not linked to the other exonuclease genes, *uvr*A and *uvr*B as in *E. coli* (Sancar *et al.*, 1984).

Restriction endonucleases *Asn*I, *Ssp*I and *Dde*I digestion of the amplified fragment of *uvr*C gene demonstrated identical restriction patterns for all the *M. bovis* strains tested, showing that the *uvr*C gene is conserved. The highly passaged strain had two more bands with the *Dde*I enzyme, showing some genetic variability in the highly passaged strain, which needs to be determined. It might be possible that the heterogeneity in the highly passaged strain was because of base pair differences as a result of mutation in this isolate. The oligonucleotide primer pair is species-specific because there was no amplification of the *uvr*C gene with either primer pair with the negative control species *M. bovigenitalium*. RFLPs have been previously reported for other genes in several other *Mycoplasma* species, the most studied being the 16S ribosomal RNA genes (Garcia *et al.*, 1995; Sasaki *et al.*, 1996). An alternative explanation for the differences observed in restriction fragments is that DNAs may have differential methylation of cytosine residues, but this would appear to be unlikely since mycoplasma DNA is characteristically AT rich (Muto *et al.*, 1984). Jansen *et al.* (1994) have reported heterogeneity of *M. genitalium* using PCR-restriction length polymorphism on an adhesion-coding gene.

PCR-RFLP technique has been widely used for the diagnosis of mycoplasmas. Bölske *et al.* (1996) used restriction endonuclease *Pst*I on the PCR product of *M. capricolum* subsp. *capripneumoniae* for the diagnosis of CCPP while Marrow *et al.* (1990) used the RFLP technique for the identification of *M. synoviae*.

Figure 6.1 The quantification of *M. bovis* DNA by 0.7 %(w/v) agarose gel electrophoresis run at 70 volt. Lane 1 marker 1kbp; lane 2, 79 B96; lane 3, 81B96; lane 4, 82B96; lane 5, 119B96; lane 6, medium passage 119B96; lane 7, high passage 119B96; lane 8, 193B96; lane 9, 67 M98; lane 10, 135B99; lane 11, 136B99; lane 12, 137B99; lane 13, 139B99; lane 14, 142B99; lane 15, 156B99; lane 16, 5B00; lane 17, 10B00; lane 18, 12B00; lane 19 NCTC 10131.

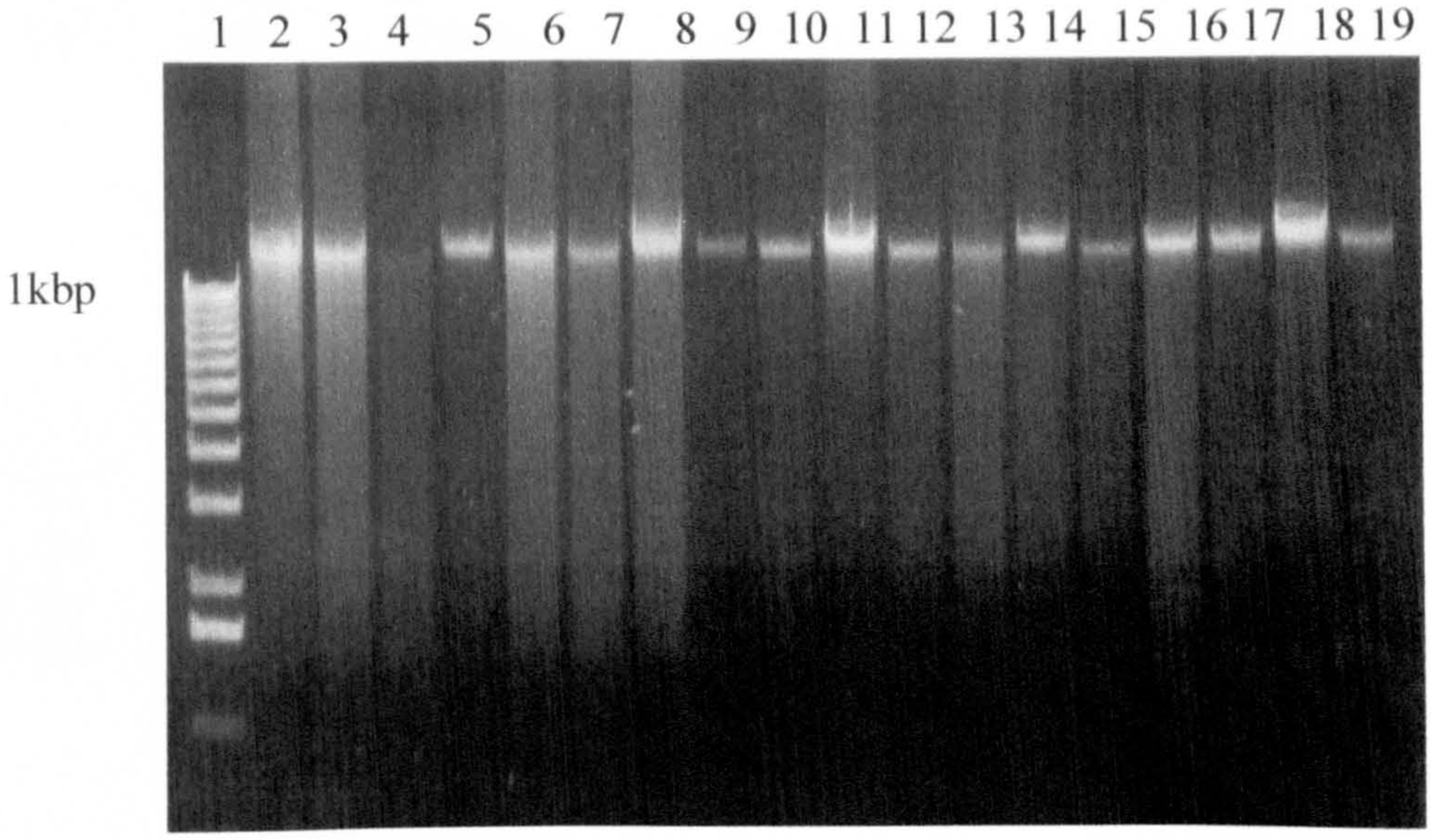


Figure 6.2 The confirmation of eighteen *M. bovis* strains by PCR. The *uvr*C gene oligonucleotide primers MBOUVRC2-L, MBOUVRC2-R that amplified 1.6-kbp product. Lane 1 marker 2kbp; lane 2, 79 B96; lane 3, 81B96; lane 4, 82B96; lane 5, 119B96; lane 6, medium passage 119B96; lane 7, high passage 119B96; lane 8, 193B96; lane 9, 67 M98; lane 10, 135B99; lane 11, 136B99; lane 12, 137B99; lane 13, 139B99; lane 14, 142B99; lane 15, 156B99; lane 16, 5B00; lane 17, 10B00; lane 18, 12B00; lane 19 NCTC 10131; lane 20 *M. bovigenitalium* (-ve control)

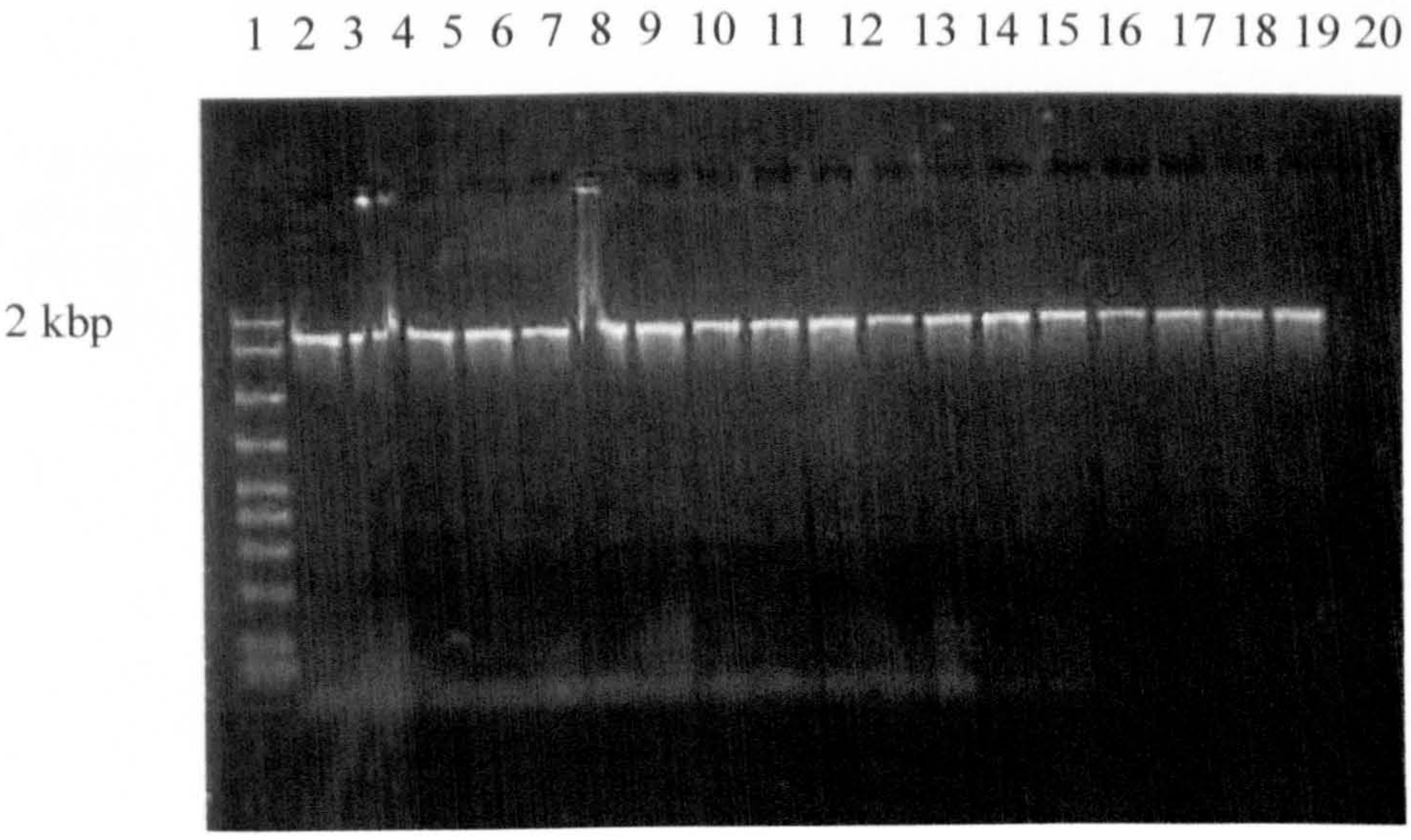
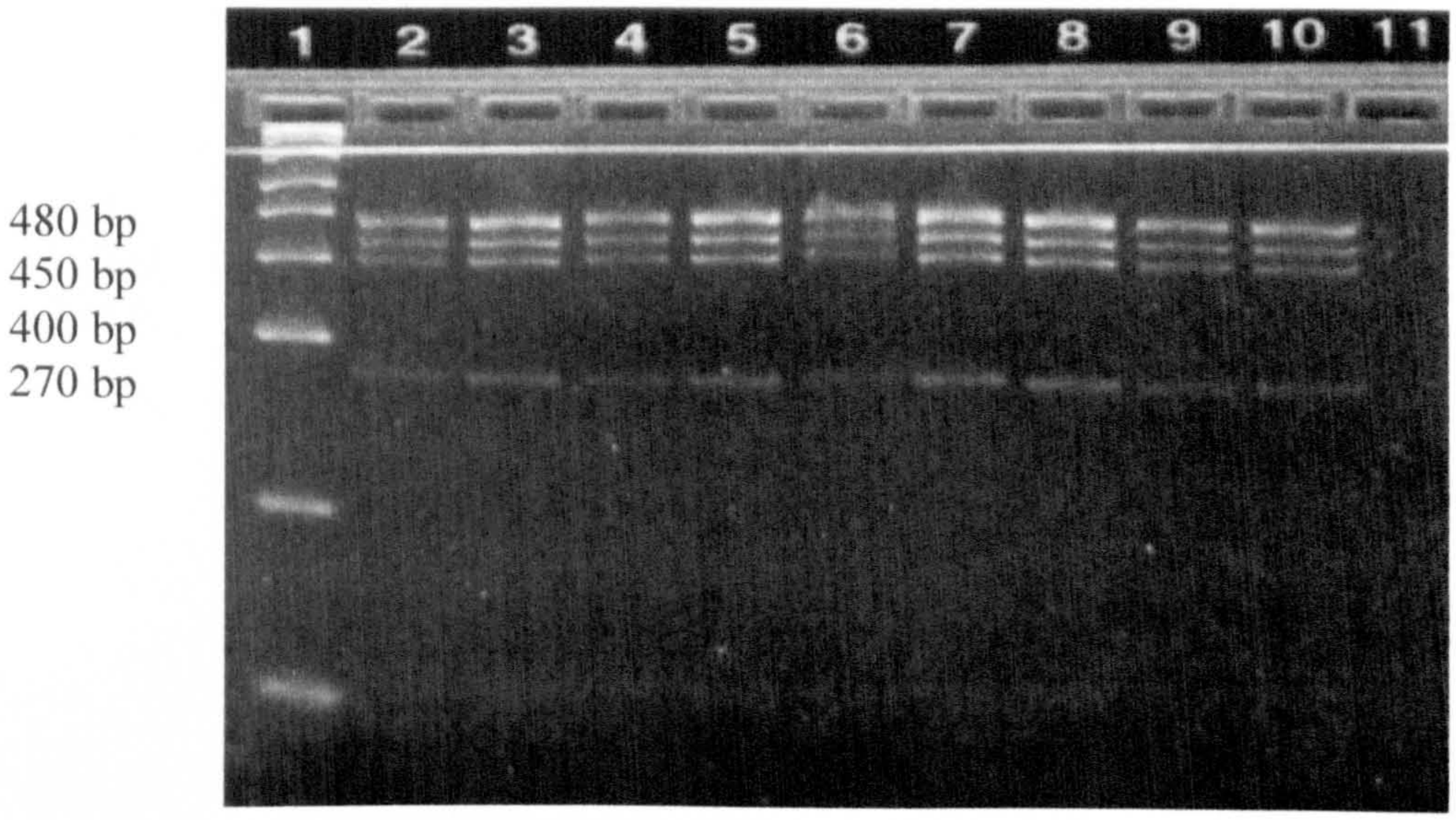


Figure 6.3 RFLP of eighteen *M. bovis* strains using restriction enzyme *Asn*I. The PCR product was digested with the restriction enzyme *Asn*I and the fragments were separated by 4% Invitrogen gel electrophoresis. A: Lane 1 marker 2kbp; lane 2, 79 B96; lane 3, 81B96; lane 4, 82B96; lane 5, 119B96; lane 6, medium passage 119B96; lane 7, high passage 119B96; lane 8, 193B96; lane 9, 67 M98; lane 10, 135B99; lane 11, blank; B: Lane 1, marker 2kbp; lane 2, 136B99; lane 3, 137B99; lane 4, 139B99; lane 5, 142B99; lane 6, 156B99; lane 7, 5B00; lane 8, 10B00; lane 9, 12B00; lane 10, NCTC 10131; lane 11, blank.

A



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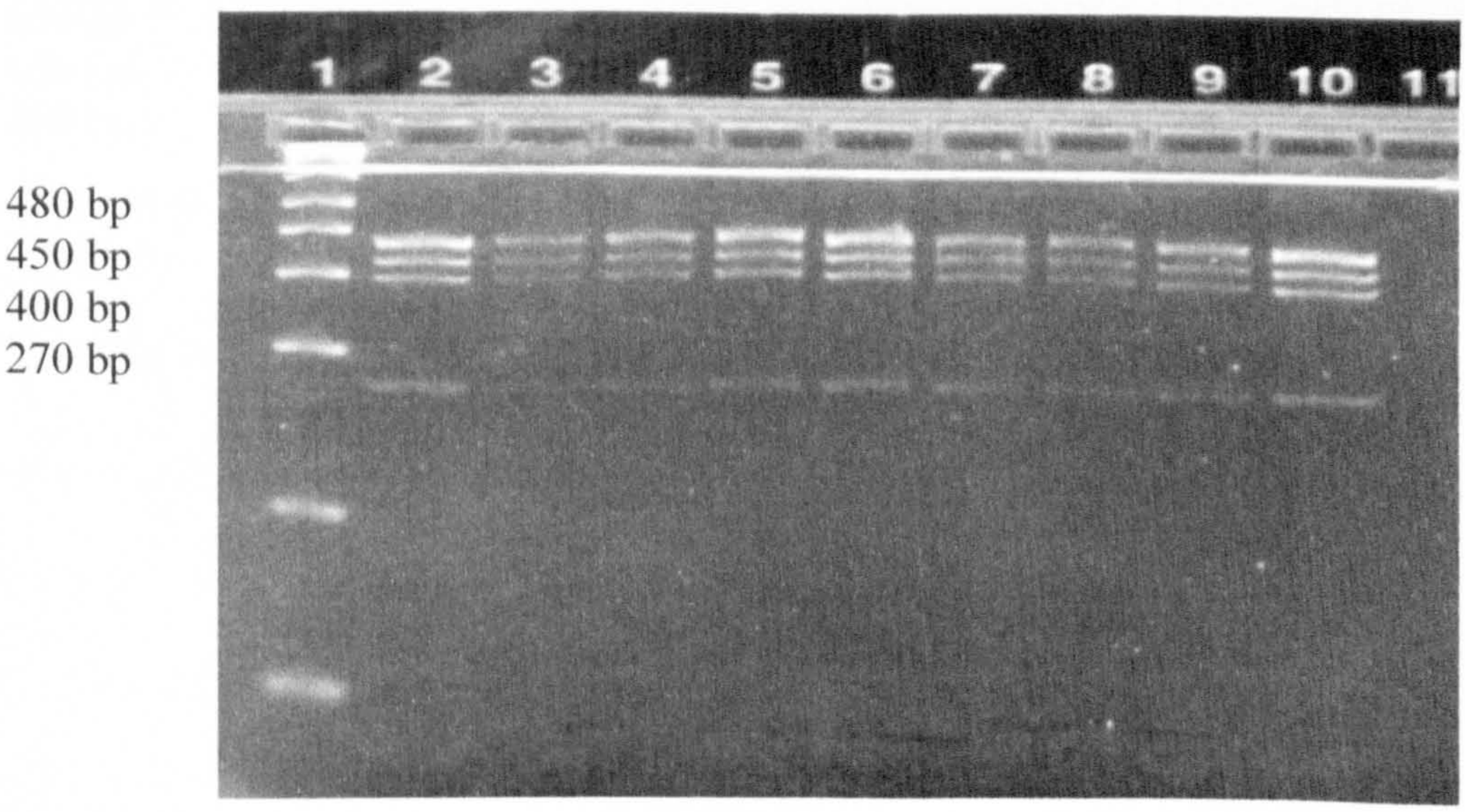
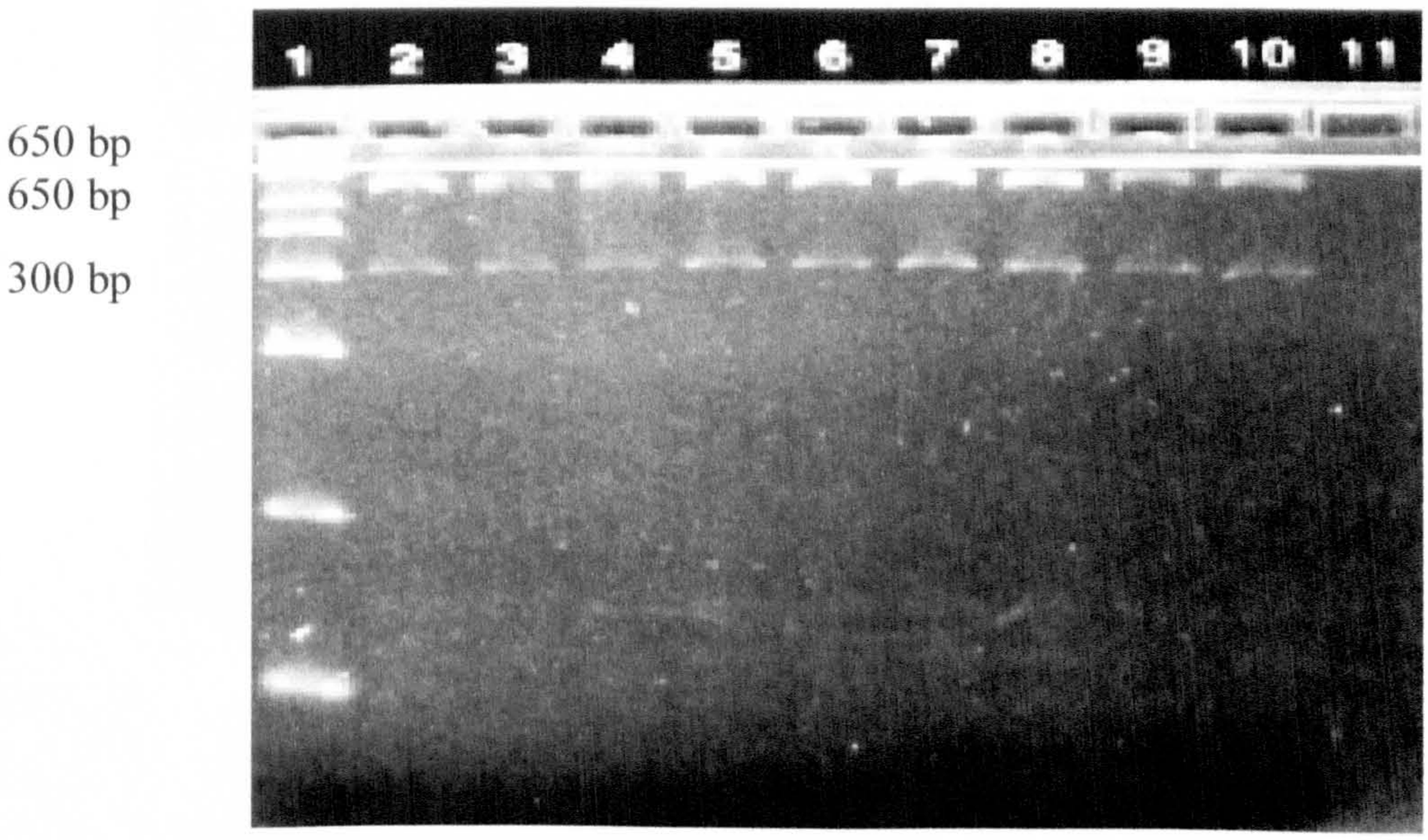


Figure 6.4 RFLP of eighteen *M. bovis* strains using restriction enzyme *Ssp*I. The PCR product was digested with the restriction enzyme *Ssp*I and the fragments were separated by 4% Invitrogen gel electrophoresis. A: Lane 1 marker 2kbp; lane 2, 79 B96; lane 3, 81B96; lane 4, 82B96; lane 5, 119B96; lane 6, medium passage 119B96; lane 7, high passage 119B96; lane 8, 193B96; lane 9, 67M98; lane 10, 135B99; lane 11, blank; B: Lane 1, marker 2kbp; lane 2, 136B99; lane 3, 137B99; lane 4, 139B99; lane 5, 142B99; lane 6, 156B99; lane 7, 5B00; lane 8, 10B00; lane 9, 12B00; lane 10, NCTC 10131; lane 11, blank.

A



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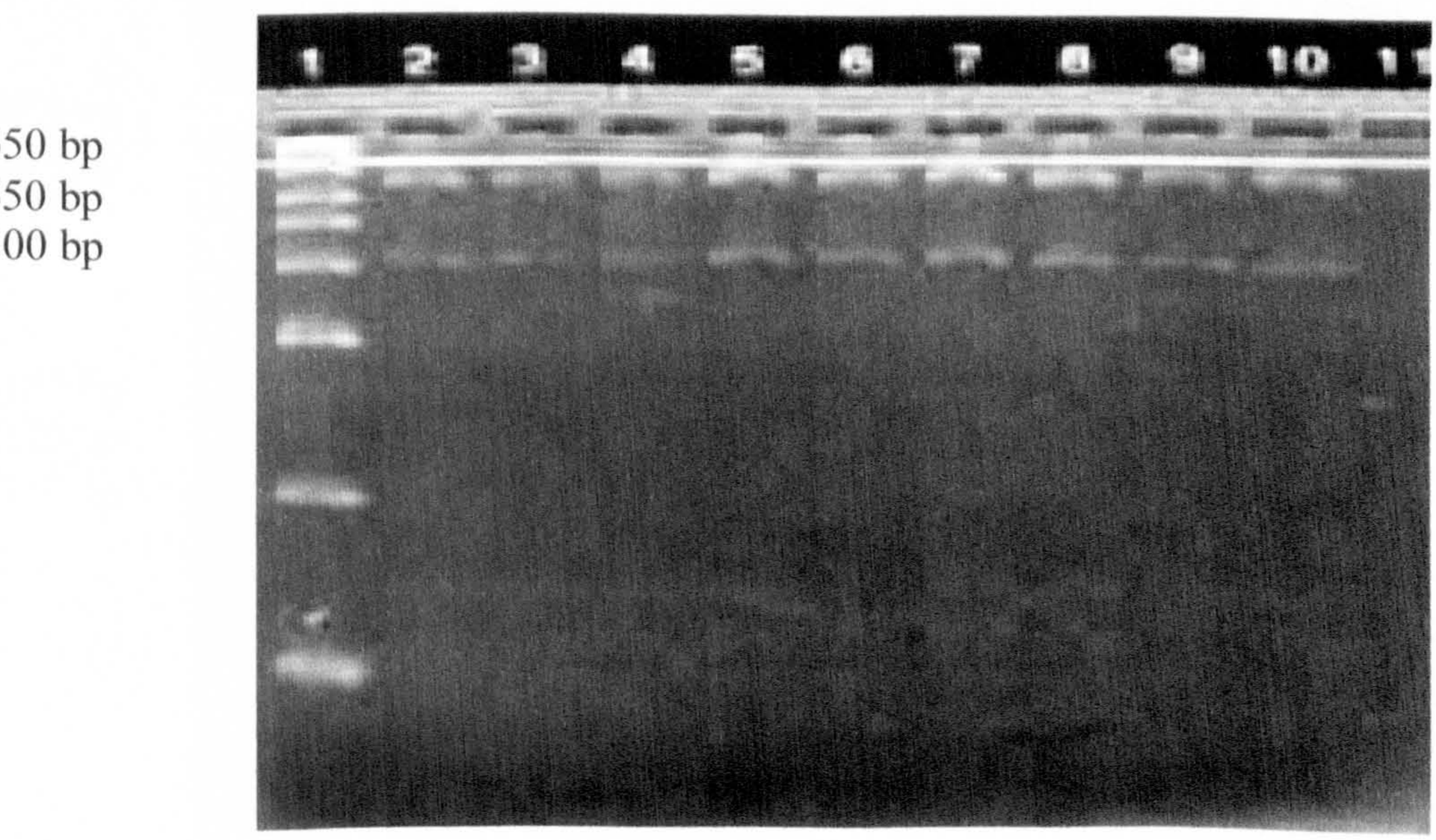
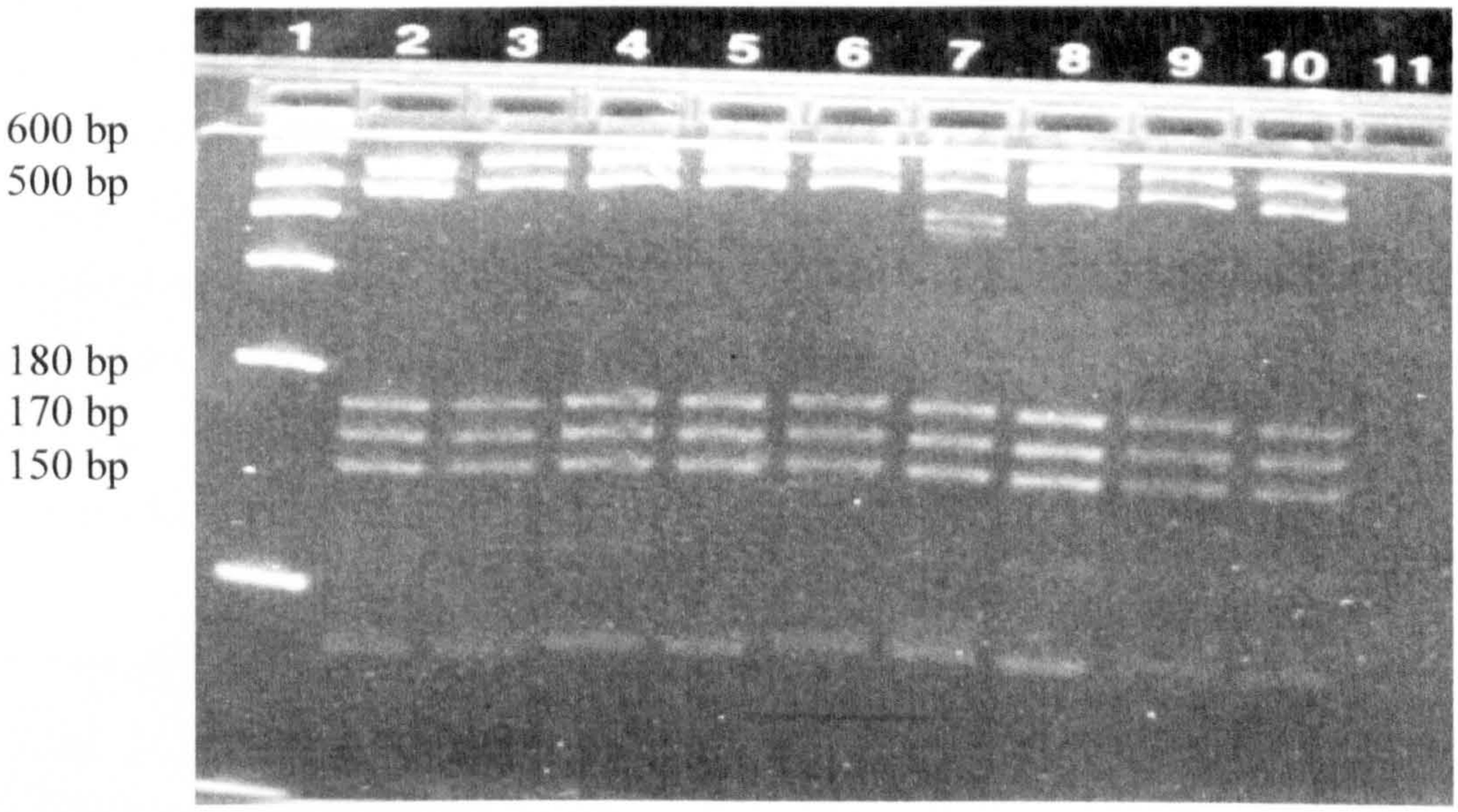


Figure 6.5 RFLP of eighteen *M. bovis* strains using restriction enzyme *Dde*I. The PCR product was digested with the restriction enzyme *Dde*I and the fragments were separated by 4% Invitrogen gel electrophoresis. A: Lane 1 marker 2kbp; lane 2, 79 B96; lane 3, 81B96; lane 4, 82B96; lane 5, 119B96; lane 6, medium passage 119B96; lane 7, high passage 119B96; lane 8, 193B96; lane 9, 67M98; lane 10, 135B99; lane 11, blank; B: Lane 1, marker 2kbp; lane 2, 136B99; lane 3, 137B99; lane 4, 139B99; lane 5, 142B99; lane 6, 156B99; lane 7, 5B00; lane 8, 10B00; lane 9, 12B00; lane 10, NCTC 10131; lane 11, blank.

A



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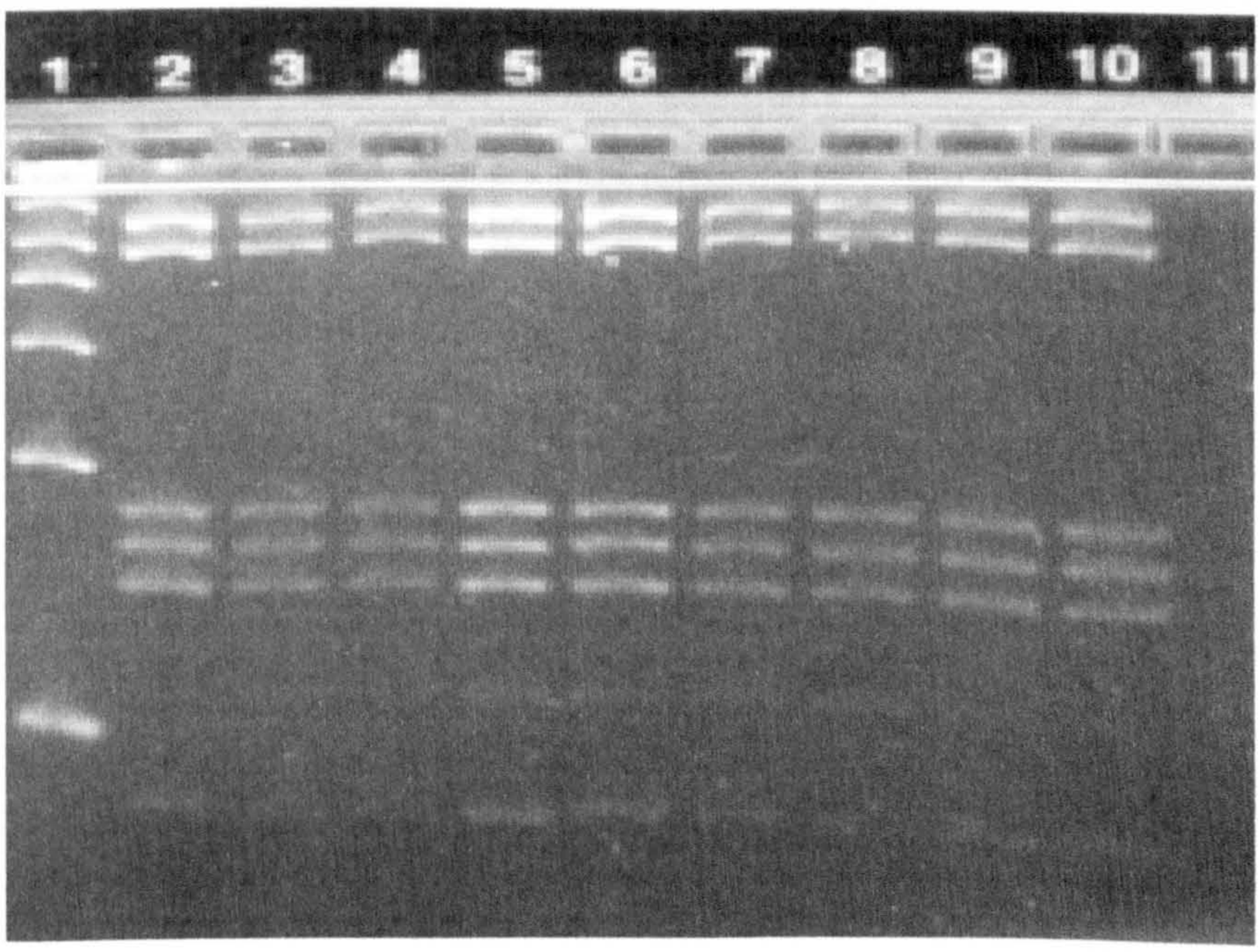


PCR-RFLP technique has been used for diagnosis, detection and identification of the avian species (Fan *et al*., 1995; Kiss *et al*., 1997).

This technique has also been used by Jarausch *et al*. (2000) for the analysis of a 1.5 kbp chromosomal DNA fragment amplified by PCR from various isolates of apple proliferation phytoplasma. The digestion of " mycoides cluster" PCR-DNA (1225 bp) amplication products with restriction enzymes *Ase*I and *Ssp*I gave *Mycoplasma* species-specific pattern for all strains of *Mycoplasma mycoides* subsp. *mycoides* LC, *M. capricolum* subsp. *capricolum* and bovine serogroup 7 (Rodriguez *et al*., 1997). PCR-restriction endonuclease system based on the 16S rRNA has been used for the diagnosis of contagious caprine pleuropneumonia (CCPP) and contagious bovine pleuropneumonia (CBPP) by Persson *et al*. (1999). On the basis of RFLP it was shown that all the *M. bovis* strains were similar and data obtained were correlated with the biochemical studies (Chapter 3), where all strains showed no consistent differences.

PCR and RFLP analysis for the detection and identification of *M. bovis* strains is validated for UK strains, and detection can be made among the amplicons by RFLP with three restriction endonucleases (Table 6.1). It is a simple, rapid and easy technique in routine mycoplasma detection and identification. The combined use of PCR and RFLP provides another tool for identifying the species of the isolate without the need for specific antisera.

Table 6.1 Identification of *M. bovis* strains by restricted fragment length polymorphism (RFLP)

Strains	**Restriction enzyme**	**Number of bands**
M. bovis all strains	*Asn*I	4
M. bovis all strains	*Ssp*I	3
M. bovis all strains	*Dde*I*	6*

*All *M. bovis* strains showed identical banding pattern except highly passaged 119B96 strain, which showed two extra bands with *Dde*I.

6.2.2 Restriction enzymes digestion of whole genomic DNA of *M. bovis*

The RFLP results did not show significant differences among the *M. bovis* strains so whole genomic DNA was digested with a range of restriction enzymes using: *Bgl*1, *Bam*H1, *Hind*III, *EcoR*1, *Pst*1, *Sma*1. *Mycoplasma bovis* DNA was digested (Section 2.16) with these restriction enzymes according to manufacturer's instructions (Promega). All these enzymes were used to check their cleavage patterns, which might be useful for the identification of *M. bovis* strains. The enzymes were selected which cleave in relatively few sites to facilitate interpretation of the results.

Eighteen strains studied gave different electrophoretic patterns with *Hind*III and *EcoR*1 (Figure 6.6 and 6.7) while *Bam*HI, *Bgl*I, *Pst*I, and *Sma*I, did not digest the whole genomic DNA completely and only a few bands were revealed even when used in the highest concentration (Figure 6.8). These results are in contrast with Poumarat *et al.* (1994) who used these restriction enzymes on *M. bovis* DNA, and were able to find 13 genomic groups of *M. bovis,* but the restriction electrophoretic pattern was not shown. The restriction endonuclease patterns of whole genomic DNA digested with *Hind*III and *EcoR*I was unclear, because a large number of bands were obtained making it very difficult to observe the differences. Solsona *et al.* (1996) have used *Hind*III, *EcoR*1 *Bam*HI, *Pst*I, and *SmaI* on the DNA of *M. agalactiae* and found differences with the *pst*I profile although again no figures were shown. However Towner and Cockayne (1993) showed restriction enzymes generated complex DNA fingerprints, making it difficult to distinguish individual bands.

Since Bove and Saillard (1979) suggested the use of restriction profiles of the mycoplasma genome as a taxonomic aid, restriction enzymes have been used widely, particularly for epidemiological studies (Kleven *et al.*, 1988). The degree of intraspecies genomic variability differs widely from one mycoplasma to another (Razin *et al.*, 1983) and results may vary depending on the sampling of strains and on the choice of enzymes (Sue *et al.*, 1991).

6.2.3 Pulsed field gel electrophoresis (PFGE) profiles of *M. bovis*

PFGE was performed as previously described (Section 2.17).

Figure 6.6 Cleavage patterns of the DNA of *M. bovis* strains by *EcoR* I. The digested DNA fragments were separated by 1 % gel electrophoresis. Lane 1 marker 1kbp; lane 2, 79 B96; lane 3, 81B96; lane 4, 82B96; lane 5, 119B96; lane 6, medium passage 119B96; lane 7, high passage 119B96; lane 8, 193B96; lane 9, 67 M98; lane 10, 135B99; lane 11, 136B99; lane 12, 137B99; lane 13, 139B99; lane 14, 142B99; lane 15, 156B99; lane 16, 5B00; lane 17, 10B00; lane 18, 12B00; lane 19 NCTC 10131; lane 20 marker.

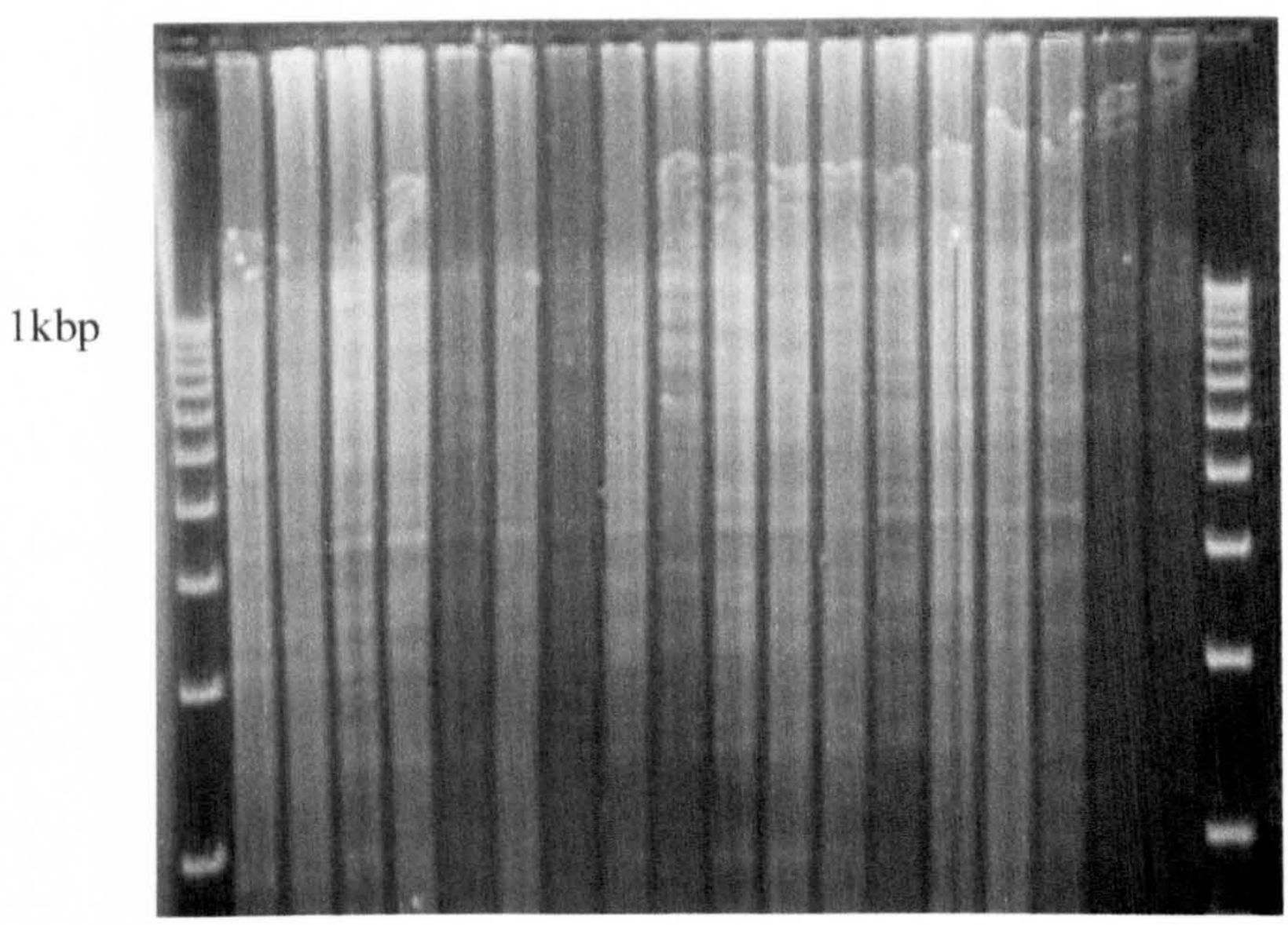


Figure 6.7 Cleavage patterns of the DNA of *M. bovis* strains by *Hind*III. The digested DNA fragments were separated by 1% gel electrophoresis. Lane 1 marker 1kbp; lane 2, 79 B96; lane 3, 81B96; lane 4, 82B96; lane 5, 119B96; lane 6, medium passage 119B96; lane 7, high passage 119B96; lane 8, 193B96; lane 9, 67M98; lane 10, 135B99; lane 11, 136B99; lane 12, 137B99; lane 13, 139B99; lane 14, 142B99; lane 15, 156B99; lane 16, 5B00; lane 17, 10B00.

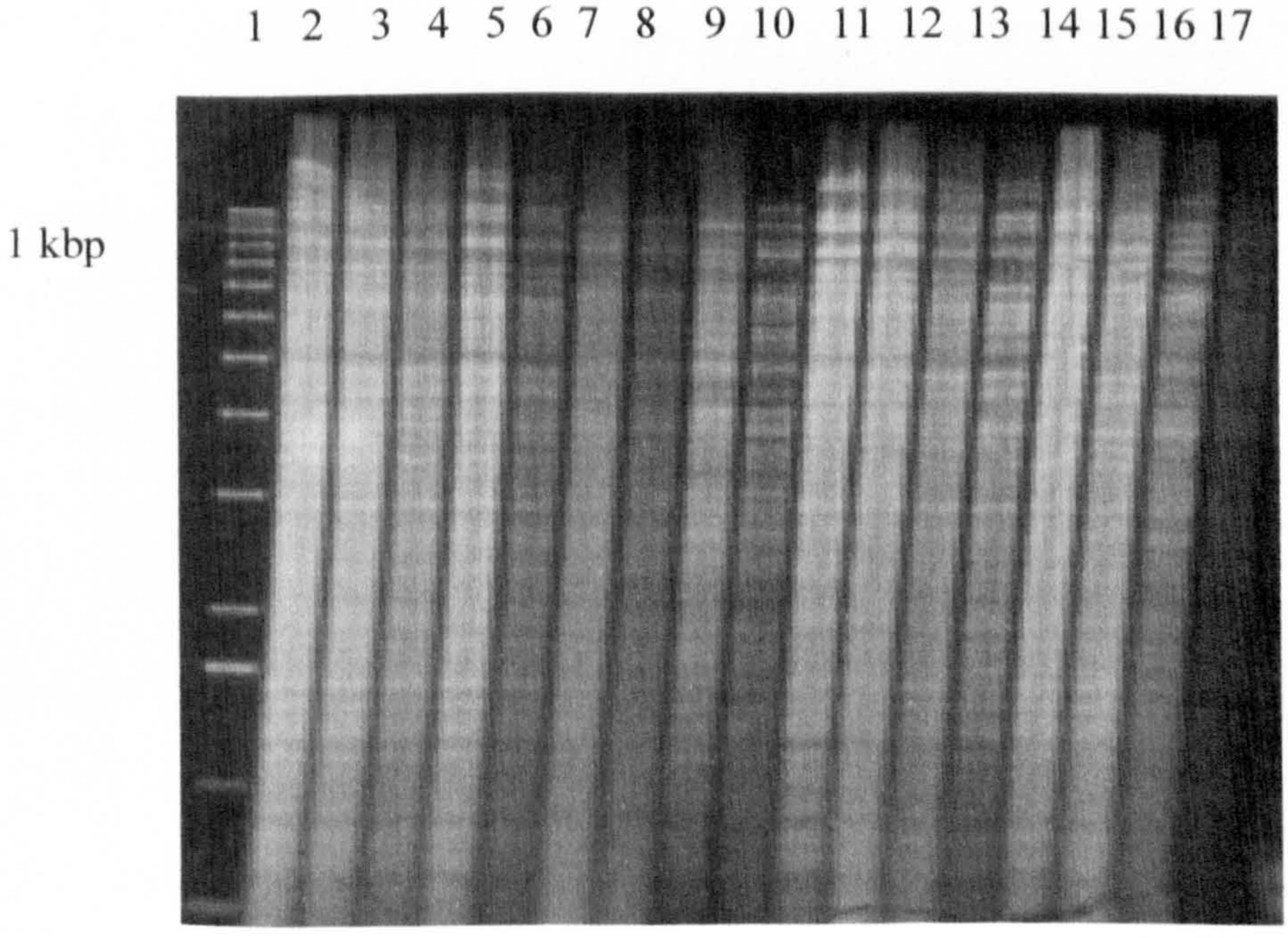


Figure 6.8 Cleavage patterns of the DNA of *M. bovis* strains by *Hind* III, *Bgl* I, *Bam*HI, *Pst*I, *Sma*I, and *EcoR*I. The digested DNA fragments were separated by 1 % gel electrophoresis overnight at 4°C. Lane 1 marker 1kbp; lane 2, 135B99; lane 3, 119B96 (RE *Hind* III), lane 4, 135B99; lane 5, 119B99 (RE *Bgl* I); lane 6, 135B99; lane 7, 119B99 (RE *Bam* H I); lane8, 135B99; lane 9, 119B96 (RE *Pst* I); lane 10, 135 B99; lane 11, 119B96 (RE *Sma* I); lane 12, 135B99; lane 13, 119 B96 (RE *EcoR* I).

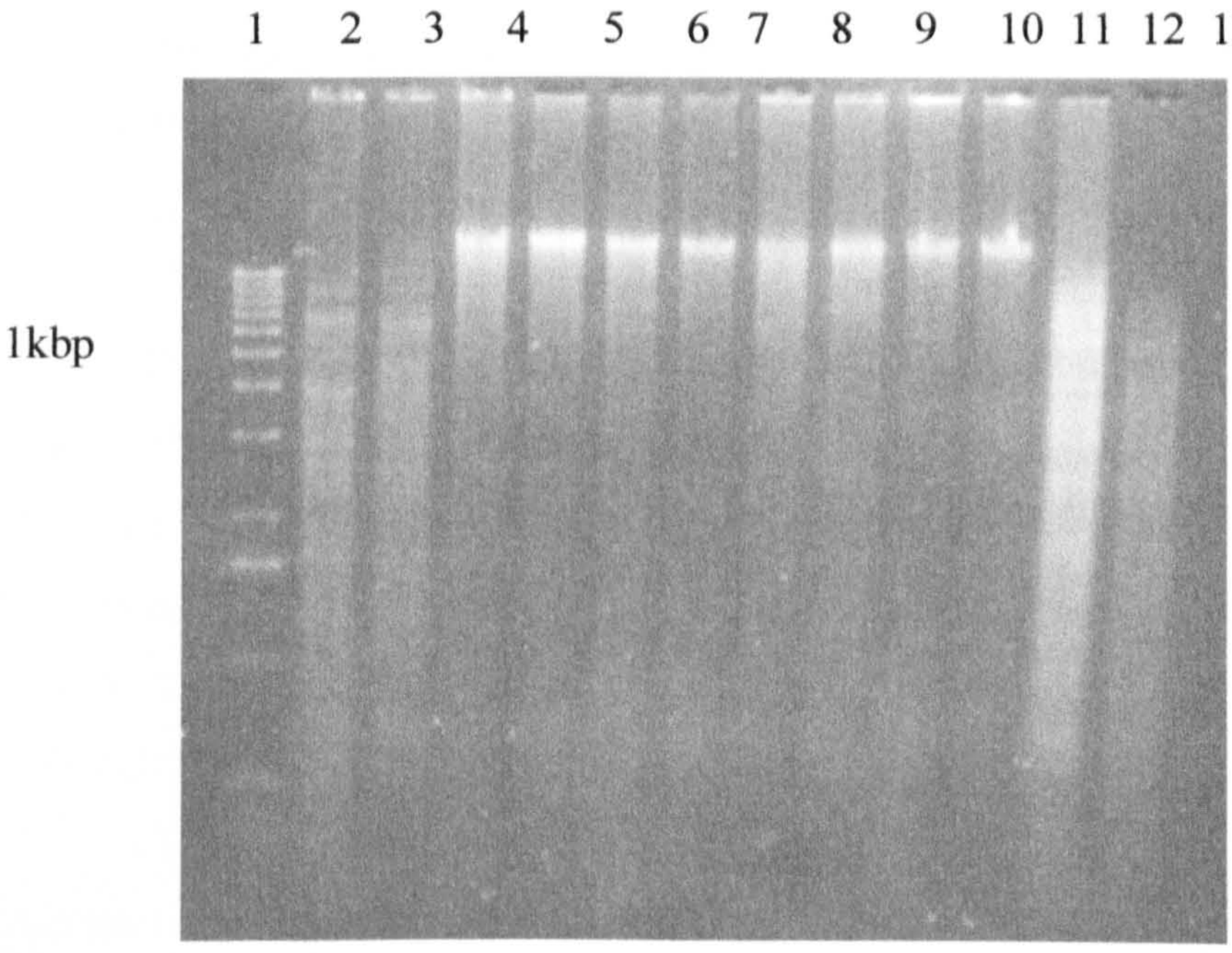
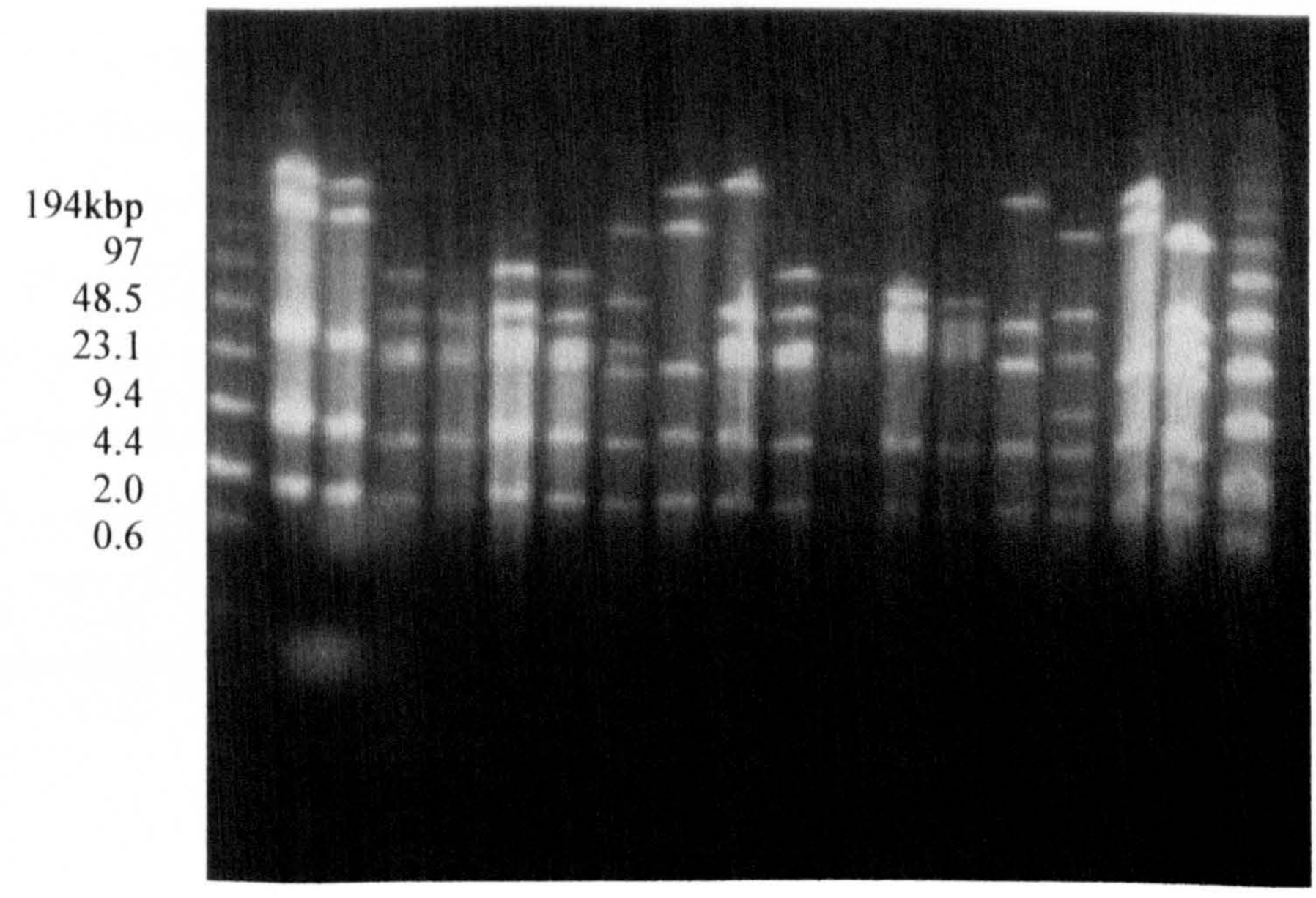


Figure 6.9 PFGE of *Sma*I digest of genomic DNA of *M. bovis* strains. Lane 1, ladder 2 kbp, lane 2-3, 67M98; lane 4, 81B96; lane 5, 82B96; lane 6, 119B96; lane 7, high passage 119B96; lane 8, 193B96; lane 9, 135B99; lane 10, 136B99; lane 11, 137B99; lane 12, 139B99; lane 13, 142B99; lane 14, 156B99; lane 15, 5B00; lane 16, 10B00; lane 17, 12B00; lane 18, NCTC 10131; lane 19, ladder 2 kbp.



The *Sma* I digest of the strains of *M. bovis* examined produced distinct DNA fragments in the size range of 0.6-190 kbp. The analysed strains produced five to seven DNA fragments comprising common and polymorphic fragments.

Strain 10B00 gave seven well-resolved DNA fragments differing from other strains. *M. bovis* strains 81B96, 82B96, 119B96 and multi-passaged strain 119B96 gave identical banding patterns. However strains 67M98 (human isolates), 193B96, 135B99, 136B99, 137B99, 139B99, 142B99, 156B99, 5B00 and 10B00, 12B00 and NCTC 10131 gave different banding patterns compared to other *M. bovis* strains (Figure 6.9). All *M. bovis* strains yielded a correlation coefficient (Daniel, 1996) of >70 % showing heterogeneity among the isolates. Reproducible PFGE profiles were obtained after three testing of identical samples. All these were field strains isolated from the same geographical location in UK. The results have revealed a remarkable genomic heterogeneity among the strains of *M. bovis* isolated from same area. This indicates that the PFGE protocol using *Sma*I may act as a useful epidemiological typing system for *M. bovis*.

Kusiluka *et al.* (2001) has used PFGE on field strains of *M. mycoides* SC and were able to discriminate field strains. However Tenover *et al.* (1995) reported that the robustness and discriminatory power of PFGE is unpredictable when fewer than 10 DNA fragments are detected. Recently PFGE technique has also been used for the analysis of *M. synoviae* (Marois *et al.*, 2001a). This technique is valuable, highly discriminatory and reproducible for molecular typing of avian mycoplasmas. Tola *et al.* (2001) have used PFGE to compare isolates and to determine *M. agalactiae* genome size. Genetic analysis indicated that isolates of *M. agalactiae* were similar without intraspecific differences (Tola *et al.*, 1996).

Bert *et al.* (1997) reported PFGE has proved more discriminating than random amplified polymorphic DNA (RAPD). The heterogeneity of *M. bovis* might be linked to mycoplasmas having high mutation rates, a feature postulated to account for marked genotypic and phenotypic diversity of the organisms constituting the mollicutes (Razin *et al.*, 1998). Mycoplasmas exhibit genotypic variability by mechanisms such as rearrangements, deletions and insertions of repetitive elements into the genome.

6.2.4 SDS-PAGE analysis of *M. bovis* strains

Whole cell proteins of sixteen strains of *M. bovis* protein were separated by SDS-PAGE (Section 2.18). All the strains showed discrete bands corresponding to molecular weights of 21-200 kDa (Figure 6.10 a, b, and c). Their comparison revealed a high degree of similarity among all *M. bovis* strains.

The majority of the isolates could be distinguished by the presence of relatively intensive band clusters and making it very difficult to detect any significant differences in polypeptide profiles. These results were similar to previous biochemical finding (Chapter 3) in which all *M. bovis* strains tested exhibited the same biochemical characteristics.

The close resemblance between the polypeptide profiles of strains could be connected with the fact that all these strains were isolated from the same bovine population of UK. These results were in agreement with Sachse *et al.* (1992) and Poumarat *et al.* (1994) who showed a high degree of similarity among the *M. bovis* strains, and strain-to-strain differences were confined to quantitative variations of certain bands.

The analysis of SDS-PAGE profiles indicated that there was no intraspecies heterogeneity, but this polypeptide homogeneity of *M. bovis* was in contradiction to immunoblotting. A similar phenomenon has already been reported for other *Mycoplasma* species: *M. hominis* (Anderson *et al.*, 1987), *M. arthritidis* (Stadlander and Watson, 1992) and *M. gallisepticum* (Avakian *et al.*, 1991). The difference was only shown quantitatively with certain bands. The number of proteins recognised in one-dimensional electrophoresis patterns of different strains comprised only one tenth of that expected from the coding capacity of an average size mycoplasma genome (Razin, 1985). Nevertheless, one-dimensional SDS-PAGE polypeptide patterns of *M. bovis* isolates can be both essential for strain identification and useful for intraspecies comparison of protein composition.

SDS-PAGE polypeptide patterns within species are known for many prokaryotes and have also been reported for *M. gallisepticum* (Khan *et al.*, 1987), *M. ovipneumoniae* (Mew *et al.*, 1985), *M. mycoides* (Costas *et al.*, 1987) and other mollicutes. *M. hominis* and *M. arthritidis*, which appear to be homogeneous when tested by one-dimensional

PAGE, showed some variability when tested by the more discriminating two dimensional PAGE technique (Watson *et al.*, 1988; Stadlander and Watson, 1992).

6.2.5 SDS-PAGE analysis of highly passaged *M. bovis* strain 119B96.

SDS PAGE was performed as described in section 2.18. The samples were subjected to gel electrophoresis and were run at 70 volt overnight. SDS-PAGE separation of whole cell proteins of the low, medium, and highly passaged strain showed significant differences. A major band of 28 kDa polypeptide was missing from all strains after 200 passages (Figure 6.11) indicating that passaged strain might have mutated. These results are also in agreement with results previously obtained (Chapter 4) in which highly passaged *M. bovis* strain showed negligible H_2O_2 production after it was repeatedly passaged *in vitro*. Chirino and Prescott (1987) obtained similar results when *Rhodococcus equi* was repeatedly passaged *in vitro* up to 100 times a polypeptide of 17.5 kDa was lost.

M. gallisepticum exhibited reduced virulence for both chickens and turkeys when passaged 10 times in each species; electrophoretic polypeptide patterns of passaged strains by SDS-PAGE showed differences (Evans *et al.*, 2000). Lin and Kleven (1984) showed that aerosol vaccination with highly passaged F or S6 strain provided good immunity against a *M. gallisepticum* challenge and that *in vitro* passages in artificial medium of a particular *M. gallisepticum* strain affected its virulence. Furthermore, experimental infection studies with chickens showed that a low passaged and a high passaged population of the *M. gallisepticum* prototype strain R colonised the trachea, while low passage induced only air sac lesions (Levisohn *et al.*, 1986). Florian *et al.* (2000) showed similar findings with low passage strains, which were capable of active cell invasion, while a high passage population showed adherence but almost no uptake into HeLa-229 cells. The invasive capability of *M. gallisepticum* was altered by serial multiplication of the original strain R in artificial media. Comparison by SDS-PAGE of polypeptide profiles of low and high passage revealed variation in several components (Lugmair *et al.*, personal communication). Baseman *et al.* (1982) reported the isolation of *M. pneumoniae* that retained their cytoadherent property, but could not invade. Extracellular material of *M. dispar* is lost upon repeated passage in culture medium (Almeida and Rosenbusch, 1991). Pathogenicity of mycoplasmas is usually altered by extended passage in unusual hosts, in only one of two alternate hosts, or in culture media.

Figure 6.10 a SDS-PAGE (12.5%) pattern of whole cell protein from *M. bovis* strains (5 μg protein loaded per track). Lane 1, prestained marker; lane2, NCTC 10131; lane 3, 67M98; lane 4,79B96; lane 5, 81B96; lane 6, 82B96; lane 7,119B96.

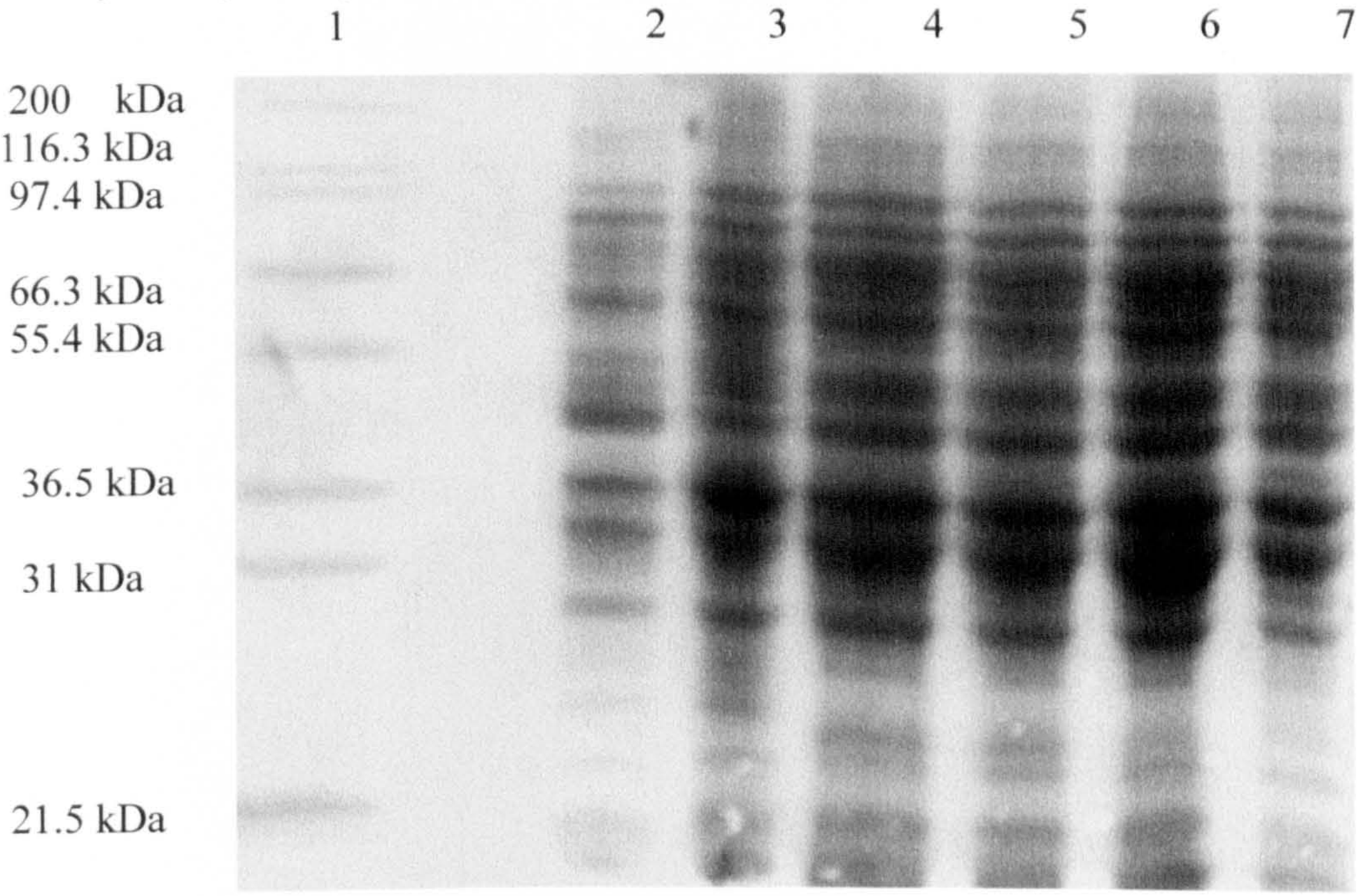


Figure 6.10 b SDS-PAGE (12.5 %) pattern of whole cell protein from *M. bovis* strains (5 μg protein loaded per track). Lane 1, prestained marker; lane2, NCTC10131; lane3, 193B96; lane 4,135B99; lane 5, 136B99; lane 6, 137B99; lane 7, 139B99.

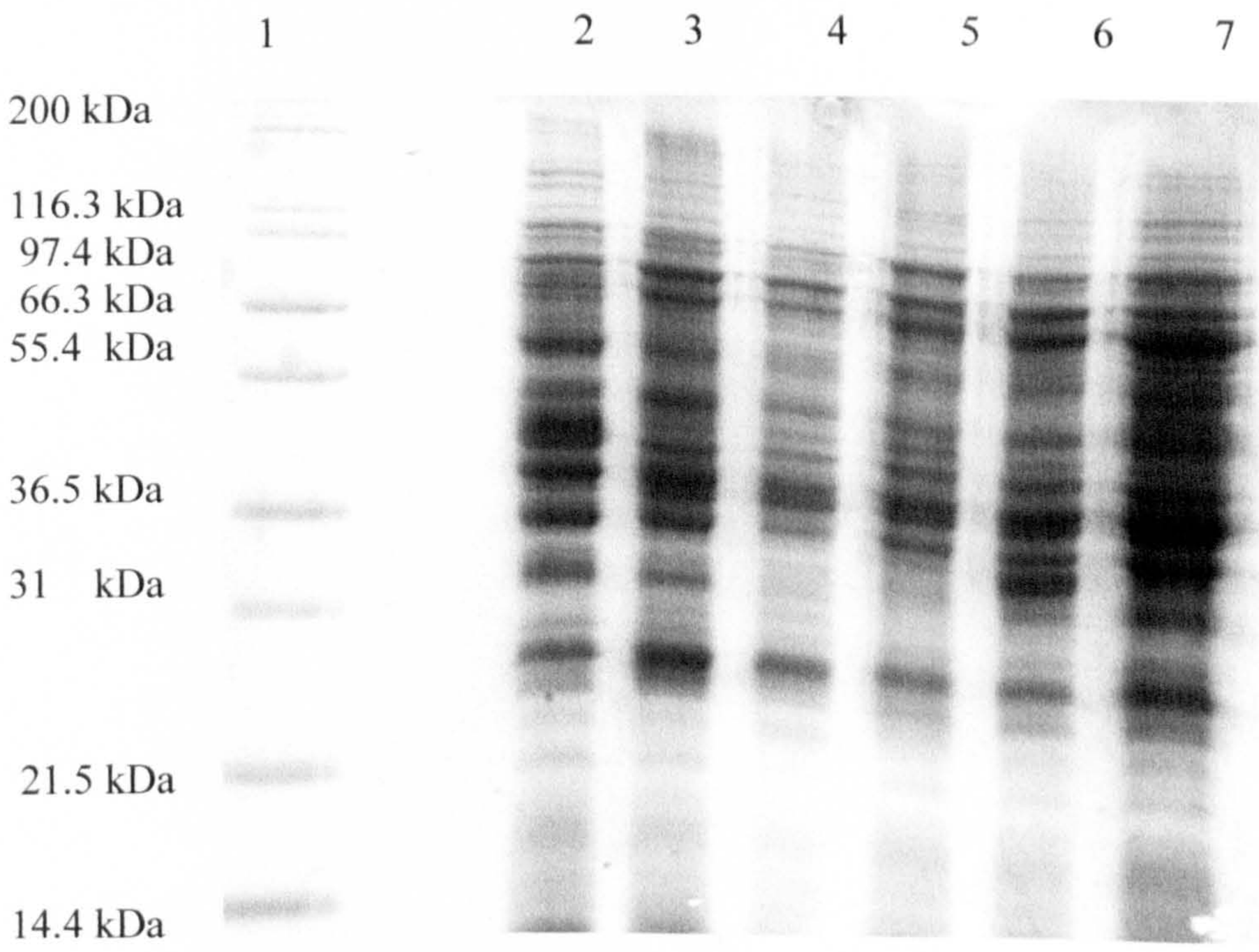


Figure 6.10 c SDS-PAGE (12.5 %) pattern of whole cell protein from *M. bovis* strains. (5μg protein loaded per track). Lane 1, prestained marker; lane2, NCTC 10131; lane 3, 142B99; lane 4, 156B99; lane 5, 5B00; lane 6, 10B00; lane 7, 12B00.

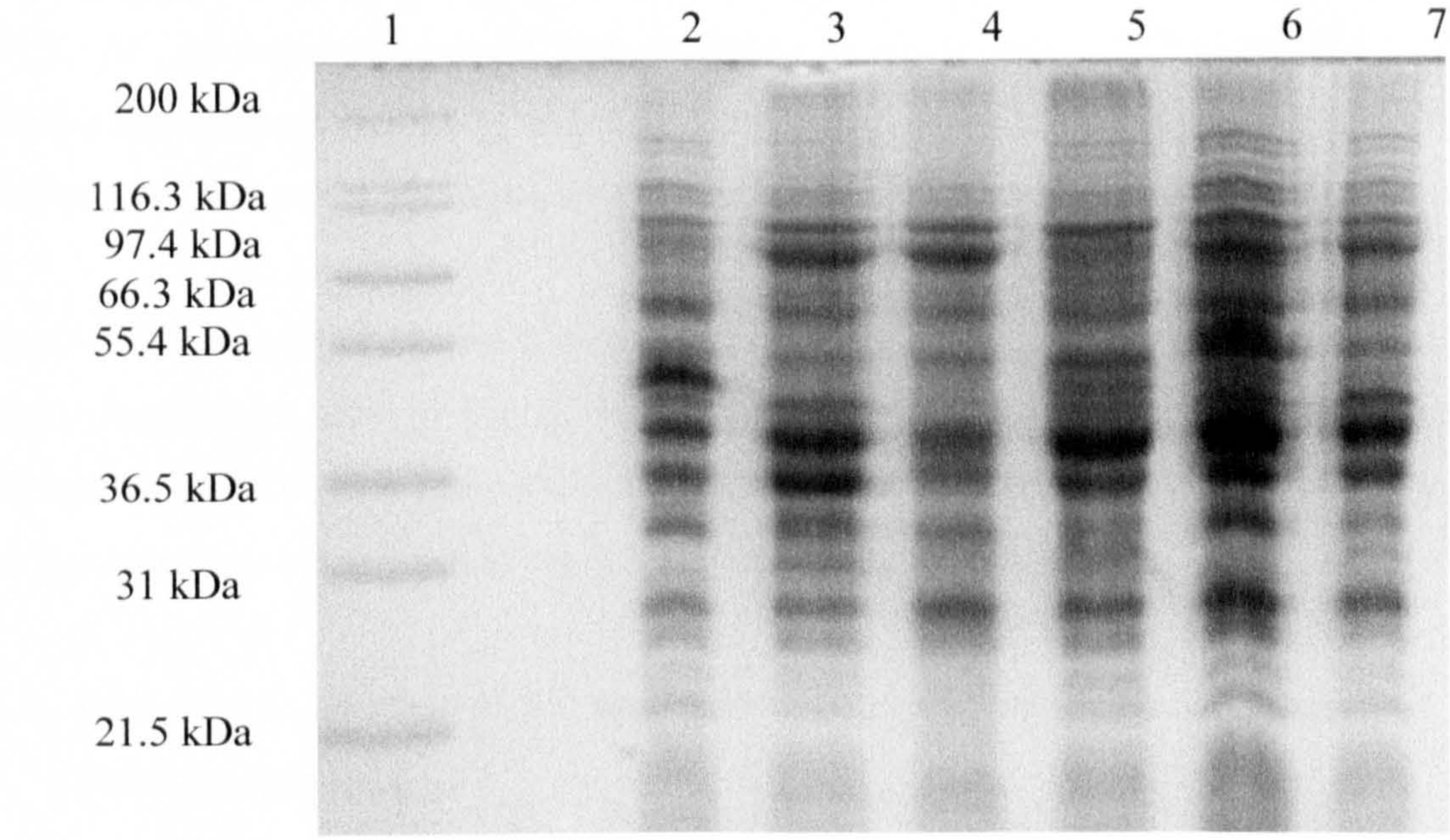
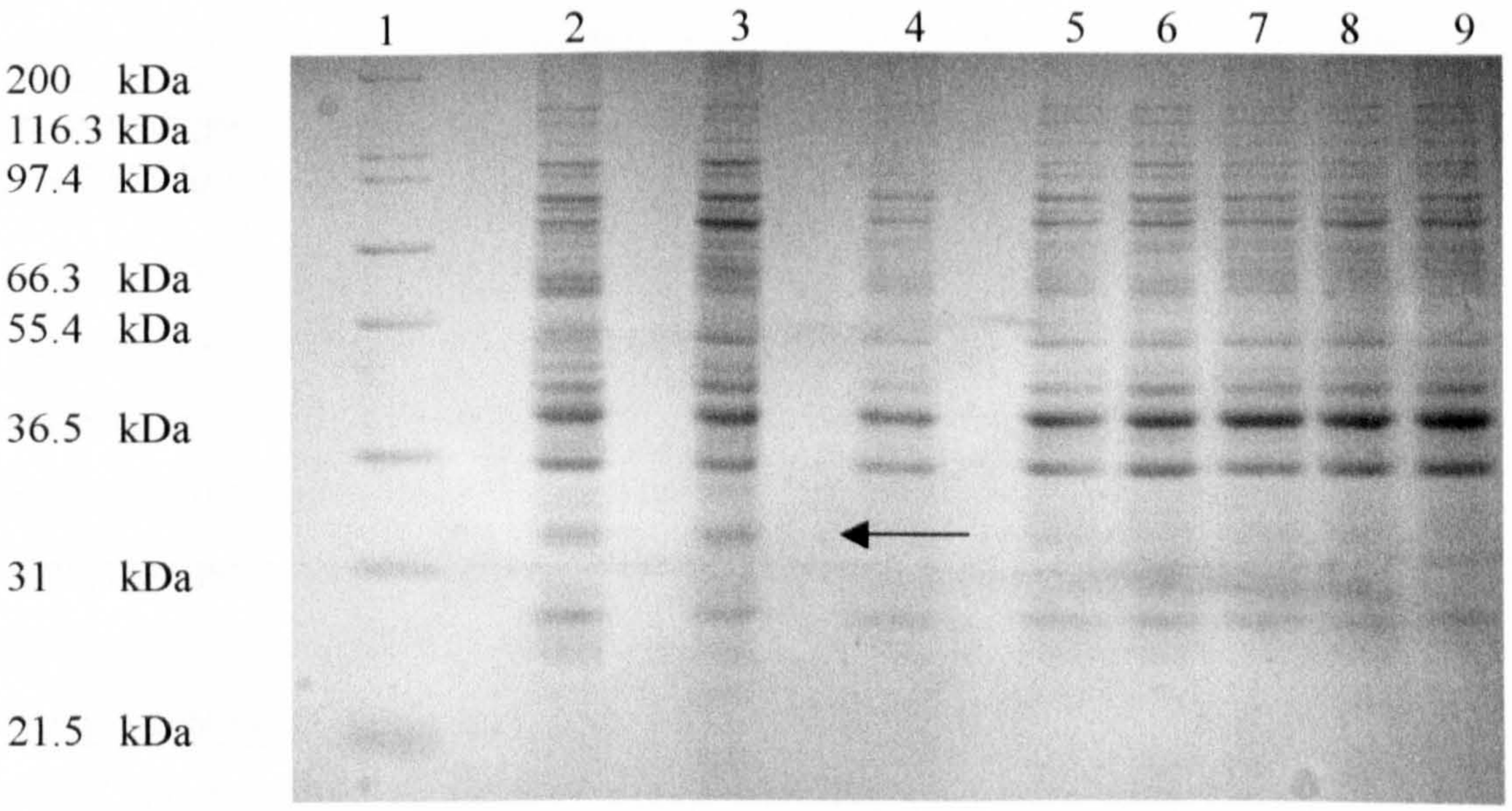


Figure 6.11 SDS-PAGE (12.5 %) pattern of whole cell protein from *M. bovis* 119B96 passage strains (20 μg protein loaded per track). Lane 1, prestained marker; lane2, NCTC 10131; lane3, 119B96 passage 0; lane 4, passage 50; lane 5, passage 70; lane 6, passage 100; lane 7, passage 110; lane 8, passage 150; lane 9, passage 200.



Dyson and Smith (1976) used a mouse virulence test to study the effect of serial passages in chick embryo or in mice. They showed that *M. mycoides* strain was readily attenuated by repeated subculture in broth. It was shown that low broth passaged strain of S6 *M. gallisepticum* caused high mortality in newly hatched chickens and turkey poults, high embryo mortality, and rapid suppression of ciliary activity in tracheal organ cultures, while the high broth passage had less effect (Power and Jordan, 1976).

Finch and Howard (1990) and Thomas *et al.* (1991) reported that a highly passaged *M. bovis* strain was able to inhibit bovine PBMC's lympho-proliferative response to mitogens. Spicer (1976) has shown increased antibiotic sensitivity following passage of *Pseudomonas aeruginosa* in carbencillin-containing media.

6.3 SDS-PAGE profiles of *M. agalactiae, M. bovis, M. bovigenitalium* and *M.* ovine serogroup11.

SDS-PAGE was performed as previously described (Section 2.18). *M. agalactiae* strains tested were NCTC 10123, 453/94, 101/94, 2123/91, 432/98, LF/00 and 499/93. All strains showed bands from 21 kDa to 200 kDa. Apart from minor differences in intensity and position of some bands, all strains showed similar profiles (Figure 6.12) although some polypeptides were quantitatively dominant. These results were in agreement with Tola *et al.* (1999) who found similar polypeptide profiles of *M. agalactiae* within the species. Solsona *et al.* (1996) also found a similar polypeptide profile for *M. agalactiae* strains.

Type and field strains of *M. agalactiae* NCTC 10123, 453/94, 2123/91, 101/93 and *M. bovis* NCTC 10131, 82B96, 139B99 were compared using SDS-PAGE. There was no significant difference in the polypeptide profile of both species, although some differences were seen in the *M. bovis* NCTC strain with bands from 36.5 kDa to 21.5 kDa having lower intensity (Figure 6.13). Whole cell proteins of *M. bovis* NCTC 10131, 142B99; *M.* ovine serogroup 11 2D, 12SR99; *M. bovigenitalium* NCTC 10122, 398/87 and *M. pullorum* 50SR99 strains were analysed using SDS-PAGE. All strains except *M. pullorum* had previously been shown to have similar biochemical characteristics. These strains showed differences in their polypeptide profile.

Figure 6.12 SDS-PAGE (12.5 %) pattern of whole cell protein from *M. agalactiae* strains (5 μg protein loaded per track) Lane 1, prestained marker; lane2, *M. agalactiae* NCTC 10123; lane 3, 453/94; lane 4, 101/94; lane 5, 2123/91; lane 6, 432/98; lane 7, LF/00; lane 8, 499/93.

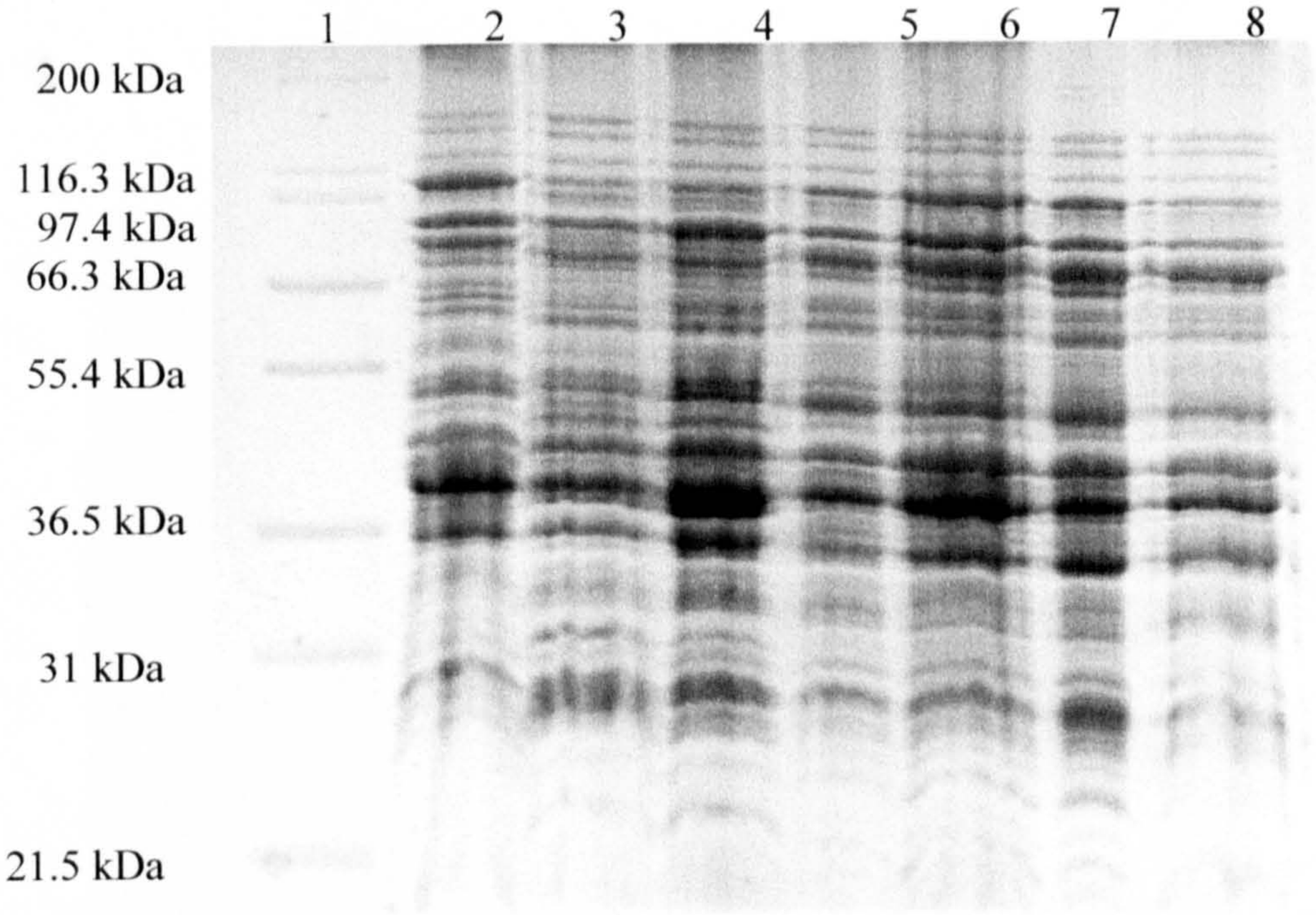


Figure 6.13 SDS-PAGE (12.5 %) analysis of *M. agalactiae* and *M. bovis* strains whole cell protein (5μg protein loaded per track). Lane 1, prestained marker; lane 2, *M. agalactiae* NCTC 10123; lane3, 453/94; lane 4, 2193/91; lane 5,701/93; lane 6, *M. bovis* NCTC 10131; lane7, 82B96, lane 8, 139B96.

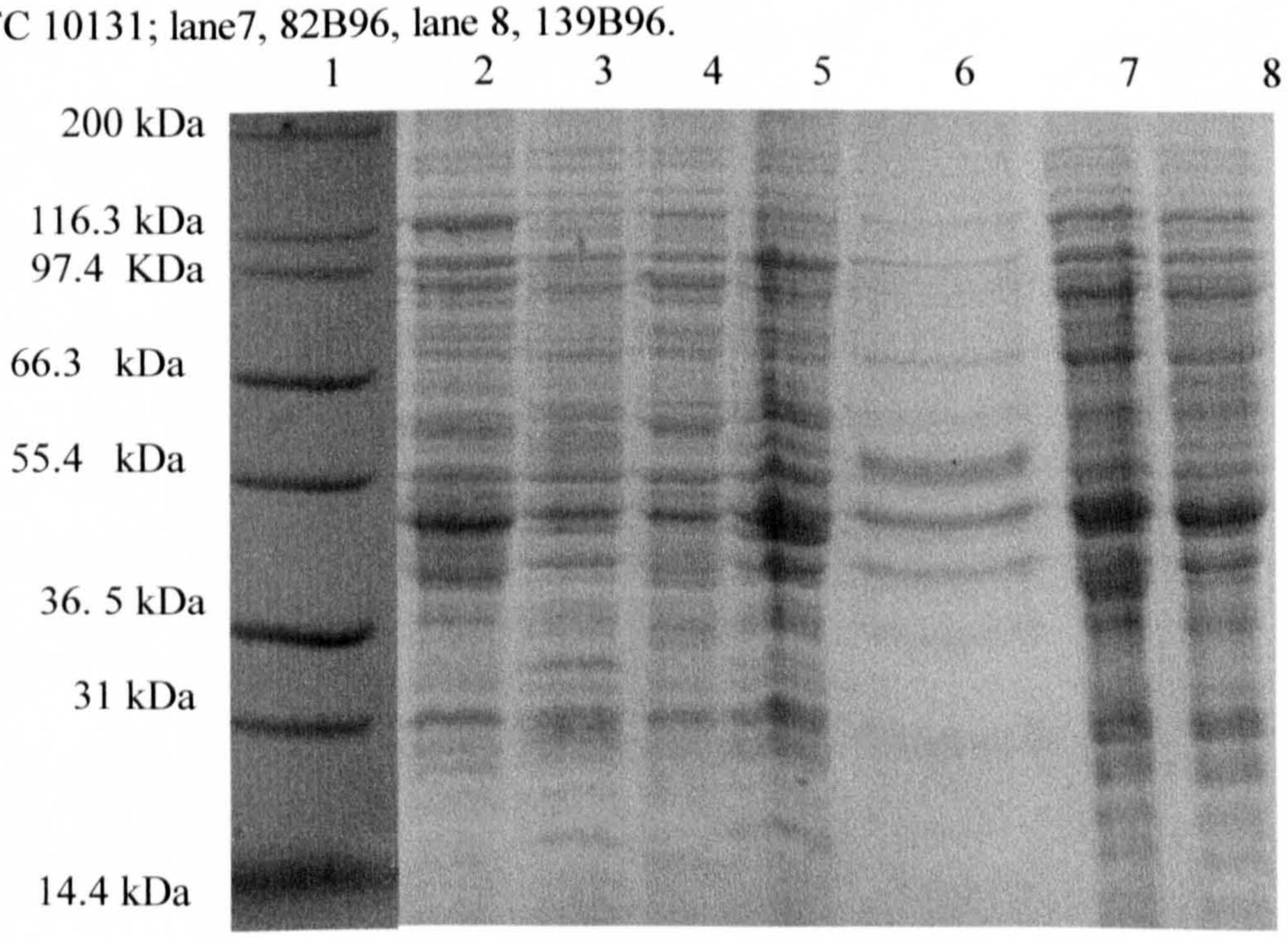
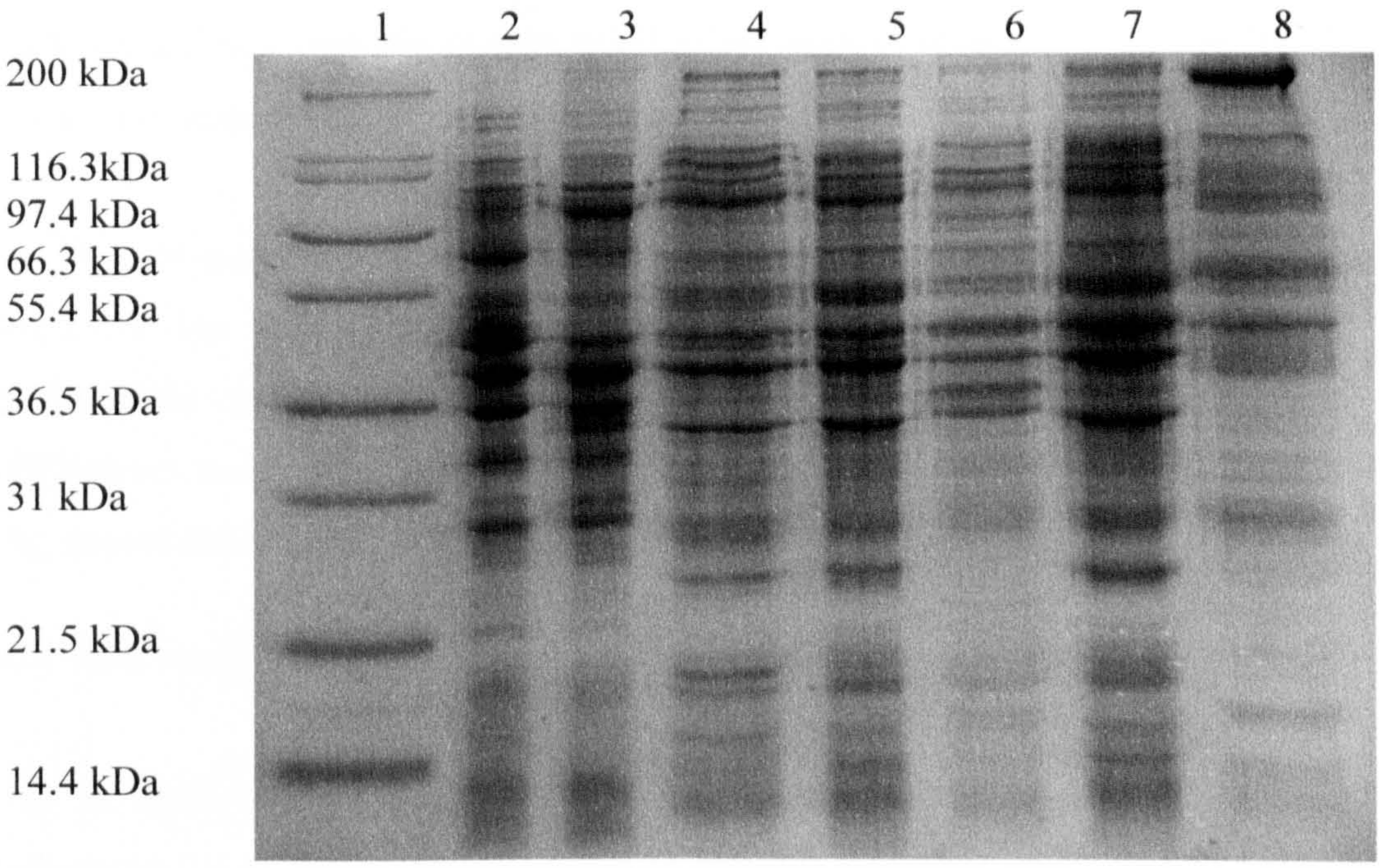


Figure 6.14 SDS-PAGE (12.5 %) analysis of *M. bovis* and *M.* ovine serogroup 11 and *M. bovigenitalium* strains whole cell protein (5 μg protein loaded per track) Lane 1, prestained marker; lane 2, *M. bovis* NCTC10131; lane 3, 142B99; lane 4, 2D; lane 5, 129B99; lane 6, *M. bovigenitalium* NCTC; lane 7, 398/87; lane 8, *M. pullorum*, 50SR99.



M. bovis showed very low intensity band of 200 kDa, while this band was very prominent in *M.* ovine serogroup 11 and *M. bovigenitalium*. *M. pullorum* showed a single major band, which was not found in any other strain (Figure 6.14). *M. bovis* showed a very intense 36 kDa polypeptide, which was not clear in all other strains except *M. bovigenitalium* NCTC 1022. *M. bovis* also showed a dominant 30 kDa polypeptide that was less intense in all other strains. *M.* ovine serogroup 11 showed a 21 kDa dominant polypeptide but was not seen in any other strains.

A 18 kDa polypeptide was not very clear in *M. bovis, M. bovigenitalium* and *M. pullorum* but was seen in *M.* ovine serogroup 11 strain 2D and 129SR99. The polypeptide profiles differences were seen intraspecies however no significant differences were found interspecies. Although biochemically these strains did not show significant differences.

6.4 Immunoblotting of *M. bovis*

All *M. bovis* strains were tested by immunoblotting using pooled sera from cattle affected with *M. bovis* (Section 2.19) to identify major antigens. The number of bands identified by bovine antiserum was a maximum of twenty (Figure 6.15). All these strains were isolated from same geographical origin in UK (Table 2.1). A dendrogram derived from immunoblot data is presented in Figure 6.15. All *M. bovis* strains exhibited antigenic similarity between 40-80 %, which revealed inter-strain polymorphism. Some bands in both the groups were immunodominant and some were weak and overall profiles were heterogeneous. This evidence obtained in this study will contribute to a better understanding of the antigenic potential of *M. bovis* as a species.

These results were in agreement Poumarat *et al.* (1994) observed marked differences in the antigenic profiles amongst *M. bovis* field strains belonging to both same and different groups. The heterogeneity being equally great among strains belonging to the same genomic groups and those coming from different genomic groups. There appeared to be no relationship between the genetic variability of *M. bovis* and the antigenic variability, unlike that observed for other mollicutes such as *Ureaplasma* (Watson *et al.*, 1988).

Mycoplasma species have shown antigenic variability when subjected to growth and metabolic inhibition tests, in particular *M. gallisepticum* (Dupiellet *et al.*, 1990) and *M. ovipneumoniae* (Jones *et al.*, 1976). Immunoblots results do not correlate with the biochemical findings, which showed no significant differences. The reasons for the antigenic variation may be that some strains were not exhibiting certain antigens or the variability could result from variations in the molecular weight of the antigen from one strain to another. This has been shown for *M. fermentans* (Stadlander *et al.*, 1991), *M. arthritidis* (Stadlander and Watson, 1992), *Ureaplasma urealyticum* (Watson *et al.*, 1988), *M. hyorhinis* (Boyer and Wise, 1989) and *M. pulmonis* (Watson *et al.*, 1988). *M. bovis* may possess a family of phase and size variable surface proteins. The successful adaptation of the mycoplasmas to the different and changing environment seems to depend on their remarkable ability to rapidly alter their antigenic surface components. In this way a diversity of organisms is maintained and probably enables the mycoplasma to evade the host immune response. *M. bovis* exhibits a high rate of antigenic variation, demonstrating that this species possess a capacity for phenotypic diversification of its surface antigenicity. This mechanism of surface diversification has also been shown in several other species of mycoplasmas (Wise *et al.*, 1992). It may allow mycoplasmas with adaptive ability to survive in a diverse range of natural habitats.

The present study showed that heterogeneity exists within *M. bovis* strains and immunoblotting may discriminate *M. bovis* isolates. This antigenic variability might be important for the development of diagnostic techniques and vaccines. Other serological techniques such as the complement fixation test have significant limitations regarding sensitivity and specificity in areas of low incidence and in disease-free areas (Regalla *et al.*, 1990). The immunoblotting test has the advantage of identifying specific antibody/antigen reactions on the basis of the molecular weight of the immunogenic protein thus overcoming the problems related to non-specific reactions in other immunoenzymatic assays. Nicholas *et al.* (1996) showed immunoblotting could be used as confirmatory test for CBPP.

6.5 Conclusions

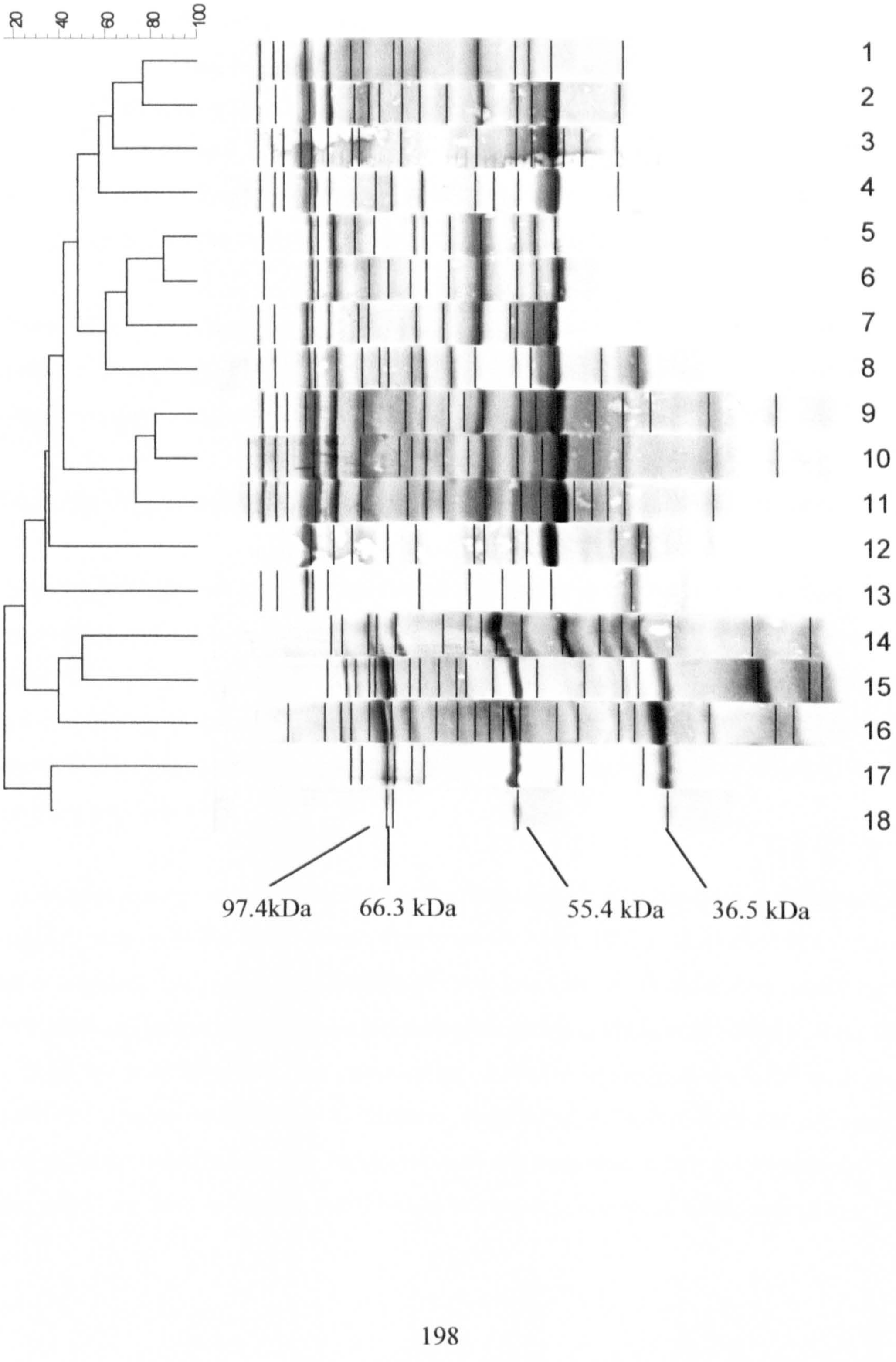
The results shown by RFLP were consistent and all *M. bovis* strains showed identical banding patterns of PCR product except *M. bovis* strain 119B96 (a multi-passage strain). The appearance of additional bands is unclear in multi-passage strain and needs

to be identified. RFLP results were consistent with biochemical studies and there were no differences found. All *M. bovis* strains were digested with restriction endonucleases and it was shown that these cleave at many sites so it was not possible to determine differences on the basis of this study. It is suggested that use of restriction enzymes on *M. bovis* whole genomic DNA is not applicable and could be approximate.

All *M. bovis* strains were analysed by PFGE, which showed discriminating banding patterns, although some strains were shown to be similar. PFGE is a more robust technique and can be used as a molecular typing of *M. bovis*. This showed ability of PFGE analysis to distinguish field isolates. Polypeptide profile of all *M. bovis* strains showed a degree of similarity among the strains, which were consistent with the biochemical studies (Chapter 3). The SDS-PAGE showed differences only in *M. bovis* strain 119B96 (multi-passage) in which a 28 kDa polypeptide band was lost and these results were correlated with the loss of production of H_2O_2, which was reduced after repeated *in vitro* passage. All the major antigens of *M. bovis* strains were identified using immunblotting technique. On the basis of this study antigenic variability was shown. The heterogeneity among the strains might be important in relation to their pathogenicity.

The polypeptide profile of *M. agalactiae*, *M. bovigenitalium* and *M.* ovine serogroup 11 was also performed. It was shown that all these strain showed identical banding pattern within the species but differences were found intraspecies.

Figure 6.15 Immunoblots of whole cell proteins of *M. bovis* strains; lane 1,79B96;lane 2, 81B96; lane 3, 82B96; lane 4, 119B96; lane 5,119B96 high passage; lane 6,193B96; lane 7, 67M98; lane 8, 135B99; lane 9, 136B99; lane 10, 137B99; lane 11, 139B99; lane 12, 142B99; lane 13,156B99; lane 14, 5B00; lane 15, 10B00; lane 16,12 B00; lane 17, NCTC 10131; lane 18, marker.



Chapter 7

7. General discussion

The major aim of this project was to determine the patterns and kinetics of substrate oxidation amongst members of non-fermentative and non-arginine-hydrolysing mycoplasmas. It was hoped that this study would enable the development of rapid biochemical tests, identification of metabolic pathways, recognition of biotypes within established taxa as well as improving the understanding their nutritional requirements, which might lead to the development of improved culture media. This information might provide insights into the study of pathogenicity in relation to toxic product formation.

A further aim was to determine whether it was possible to subdivide the most important member of this group, *Mycoplasma bovis*, using biochemical, genetic or immunological techniques which may prove useful for epidemiological puposes.

7.1 The development of biochemical procedures and improvement of medium

Mollicutes generally possess few biochemical characteristics that can be applied to the identification and classification of species (Tully, 1983). PCR methods are common but identification is based primarily upon serological tests (Bashiruddin, 1998). Serological cross-reactions amongst species are frequently observed and, for both serological and PCR techniques, the large number of species to which isolates may belong makes identification expensive and laborious.

In mollicutes, energy may be obtained by: the fermentation of sugars; the partial oxidation of organic acids and the metabolism of arginine (Pollack, 1992). Individual *Mycoplasma* species may use one or any combination of these reactions to obtain energy, enabling the subdivision of the genus into major physiological groups (Miles *et al.*, 1994). Very little has been reported regarding the presence of alcohol dehydrogenase (ADH) activity or kinetics of alcohol oxidation by mollicutes. Salih *et al.* (1983) studied the presence of thirty different enzymes in acholeplasmas and mycoplasmas using gel electrophoresis. They were only able to see the ADH bands but no description of bands was given. The

relatively low affinity of the mycoplasmas tested for alcohols (ethanol and propanol) and acetaldehyde suggest that alcohol uptake might not be via a specific membrane carrier. Small lipid-soluble molecules, including alcohols and glycerol are known to cross cell membranes at relatively high rates compared to sugars (McElhaney *et al.*, 1970). The role of ADH activity in mollicutes is not clear. All these *Mycoplasma* strains tested were adapted to isopropanol, which might be derived from anabolic metabolism within the gut. The K_s values for isopropanol were low showing high affinity for this substrate. The oxidation of isopropanol by *M. bovis* might be particularly useful as a confirmatory test and enable screening for this species when *Acholeplasma laidlawii* (non-isopropanol oxidising species) is also present in the upper respiratory tract of cattle. There is no known mechanism in mollicutes whereby energy may be derived from oxidation of alcohols. It is possible that ADH activity might be important in the reverse direction i.e. catalysing the conversion of acetate or acetaldehyde to ethanol. In yeast, the formation of ethanol as a result of glucose fermentation is dependent upon the decarboxylation of pyruvate to acetaldehyde by pyruvate decarboxylase. ADH then converts acetaldehyde to ethanol. This reaction may be reversible leading to the formation of acetaldehyde by ethanol oxidation (Goschalk, 1979). But Pollack (1992) reported that there is no evidence of pyruvate decarboxylase in mollicutes. Any ethanol formation would most likely be dependent on the conversion of pyruvate to acetyl-CoA by pyruvate dehydrogenase. Acetyl-CoA might be then reduced by acetaldehyde dehydrogenase to acetaldehyde and converted to ethanol by ADH activity. This pathway occurs in many lactic acid bacteria, enterobacteria and clostridia (Goschalk, 1979).

Patterns and rates of substrate oxidation offer an alternative procedure for identification and limiting the range of potential species for subsequent serology or PCR. These procedures may be used to distinguish certain species and sub specific groups and may also be applied to the biochemical characterisation of isolates. The only widely used biochemical tests are glucose fermentation and arginine hydrolysis. The techniques used are not quantitative and routine detection of metabolism of other substrates during mollicute growth is often not feasible because of low cell yields and difficulties in detecting the metabolism of specific substrates in complex media containing a wide range of alternative substrates. Kinetic data are important in allowing assessment of the likely significance of substrate metabolism at the concentration found in host tissues.

Patterns and kinetics of substrate oxidation were determined using washed cell suspensions. Single substrates were added which avoided the complex nature of the mycoplasma growth media. Serum has been reported to contain enzymes, which rapidly hydrolyse di and polysaccharides of glucose (Miles, 1983) and might also contain enzymes such as lactate dehydrogenase. The work presented here mainly focused on non-fermentative and non-arginine hydrolysing mycoplasmas. All strains of this group were able to oxidise organic acids and alcohols but were unable to oxidise sugars. The substrates are divided into those metabolised at low concentrations and those for which metabolism was detected only at a relatively high concentration. There was generally high affinity (low K_s value) for substrates in the first category, suggesting specific adaptation to their use. In the second category are substrates for which there was a low affinity. It is doubtful whether these substrates are ever present at high concentrations *in vivo* for their utilisation to contribute significantly to energy yielding metabolism and, possibly, cells are not specifically adapted to their utilisation. It was shown that these species possess only a part of EMP pathway. Such specialisation and the infrequent demonstration of inducible enzyme activity in these species implies adaptation to specific habitats in host species, and suggests that differences in the catabolic activities of these species may be significant in terms of their ecology and pathogenicity. The data obtained were compared with the fermentative species such as *M. mycoides* SC, isolates of which were also included in the study. All *M. mycoides* strains oxidised glucose and other sugars at high rates and were unable to oxidise glycerol except African SH9 strain, which oxidised glucose at low rate and glycerol which was oxidised at high rate.

The pattern of ethanol, acetaldehyde and propanol oxidation was complex. The addition of low concentrations caused stimulation of the oxygen uptake. The extent of stimulation increased with increasing alcohol concentrations until saturation was achieved. A possible explanation is that at low concentration substrate transport is limiting metabolism and the kinetics are influenced by the activity of a membrane-bound carrier protein, whereas at high concentration passive diffusion becomes significant. The rate of passive diffusion is proportional to the difference between the external and internal ethanol concentrations. However as the internal concentration becomes high ethanol dehydrogenase (EDH) activity may become limiting. There may also be more than one alcohol dehydrogenase activity. At high concentrations alcohols may be toxic affecting oxygen uptake rate. The results

suggest that for all these species, the alcohol uptake and/or dehydrogenase systems are specific for isopropanol rather than ethanol or propanol.

Wadher *et al.* (1990), Abu-Groun *et al.* (1994) and Taylor *et al.* (1996) have shown the potential value of pattern and kinetics of substrate utilisation in mycoplasma identification and pathogenicity. *Mycoplasma fermentans* (incognitus strain) preferred fructose to glucose, suggesting that it is adapted to a habitat(s) where fructose is available. Miles *et al.* (1994) reported the induction of fructose utilising ability of *M. mycoides* subsp. *mycoides* SC as being possibly associated with the growth of the organism in the foetus. *M. mycoides* subsp. *mycoides* SC strains are known to cross the placenta. The inability to use sugars such as N-acetylglucosamine, maltose, mannose and trehalose by non-fermentative and non-arginine hydrolysing mycoplasmas might also reflect the pathogenicity of the strains. These sugars are not present at detectable levels as free sugars in the serous fluids of animals though they are present in glycoconjugates.

M. mycoides SC utilised substrates such as glycerol, mannose and N-acetylglucosamine, which are present in much lower concentrations in mammalian serum than glucose may be important in their pathogenicity (Miles *et al.*, 1985). However sugars (N-acetylglucosamine, fructose glucosamine, and mannose) gave poorer growth than glucose; also, maximal growth rate were not increased by the addition of these sugars to medium containing glucose (Rice *et al.*, 1999). It is also possible that the utilisation of substrates such as mannose and N-acetylglucosamine reduces their concentration to extremely low levels, so promoting the enzymatic hydrolysis of oligosaccharides on glycosylated proteins. This might lead to tissue degradation and to alteration in the antigenicity of the proteins possibly leading to autoimmune phenomena which has been speculated to occur in some mycoplasma diseases like CBPP. Lysosomal-derived N-acetylglucosaminidase and other glycosidases are present on normal mucosal surfaces (Hussain *et al.*, 1992); mycoplasma appears to possess low glycosidase activity (Miles, 1992b). Furthermore glucose may have a role in fermentative species in addition to being an energy-yielding substrate, which may not be fulfilled efficiently by mannose, glucosamine or N-acetylglucosamine. This role could be in the synthesis of other sugars or polysaccharides, or in metabolic regulation. The metabolic differences detected between fermentative species (*M. mycoides* SC) and

non-fermentative species (*M. agalactiae*, *M. bovis*, *M. bovigenitalium* and *M.* ovine serogroup 11) may serve as marker when investigating their phylogenetic relationships.

The use of the oxygen electrode or pH meter to determine substrate utilisation gave reproducible and reliable data. But these techniques are not simple and need specialised equipment which cannot be widely applied in routine laboratories. Therefore a rapid chromogenic substrate test was developed as an alternative to the measurement of oxygen uptake for the detection of lipolytic activity in mycoplasmas. Chromogenic substrate have been used widely for the identification of other microorganisms, it has been used recently for the identification of *Candida* sp (Cook *et al.*, 2002). This test is robust, quick and can be used in the field for the primary identification of *M. agalactiae* and *M. bovis*. This might be useful and widely applied in routine mycoplasma preliminary identification without the need for special equipment. It is possible that the lipolytic activity in these two species might be higher than other *Mycoplasma* species tested. Rollof *et al.* (1988) described lipases as suspected virulence factors, Buttke and Cuchens (1984) showed that the end products of lipolytic activity such as free fatty acids affect several immune functions, so this activity might also be important in relation to the pathogenicity of *M. agalactiae* and *M. bovis*.

Biochemical studies provide data which can be used for the development of media for mycoplasmas and to understanding their nutritional requirements. It was expected that the rapidly oxidised substrates would be important sources of energy or metabolic precursors *in vivo*. Defined media have been described for anaerobic mollicutes (Robinson, 1979) and minimal defined media have been developed for some *Mycoplasma* species (Rodwell, 1983). Thus mollicutes are generally grown in complex media containing high concentrations of serum. Even in such media growth rate and growth yield are typically low and broth cultures of many mollicutes do not reach visible turbidity.

The data presented in this thesis have identified energy substrates for non-fermentative and non-arginine-hydrolysing species tested. It was observed that pyruvate and 2-oxobutyrate were possible sources of energy for non-fermentative and non-arginine-hydrolysing mycoplasmas. Pyruvate may be a particularly good energy source stimulating the growth rate and yield in media with or without glucose. Pyruvate may be both oxidised to acetate

(energy generating) and reduced to lactate, in an overall reaction requiring no oxygen. On the other hand, L-lactate did not increase the growth yield and was a poor energy source. Lee *et al.* (1986) have shown that lactate and pyruvate transport in *M. mycoides* involves common components. However, i*n vivo*, the role of lactate may be significant. Lactate is more plentiful than pyruvate in serum, oral and vaginal secretions and its failure to promote growth of lactate-oxidising strains may reflect oxygen limitation in statically incubated cultures. Oxygen increases the growth of *Mycoplasma* species possibly because it increases the rate of pyruvate oxidation and thus the yield of ATP during the metabolism of sugars. But excessive aeration decrease cell viability because of production of reactive oxygen species like H_2O_2. It is suggested that these strains are adapted to utilise these substrates *in vivo*. The ability to oxidise lactate at high rates has been proposed as a pathogenicity factor in, for example, *Neisseria gonorrhoea* (Britigan *et al.*, 1988), reducing the oxygen concentration and thus limiting the ability of neutrophils to generate reactive oxygen intermediates.

A number of novel vegetable peptones were tested to replace ruminant sources of peptones; vegetable peptone broth was preferred as it gave highest yield and light-coloured medium. Hence this can replace the ruminant source of peptone in PRM broth media which might pose a risk in spreading bovine spongiform encephalopathy. The cell yield obtained in PRM makes the medium suitable for the production of antigens for vaccine and diagnostic tests. Selective inhibitors are widely used in isolation for specific cell-walled bacterial groups. The identification of inhibitors acting selectively among mycoplasmas would enable similar development of media for specific groups of *Mycoplasma* sp. The availability of such media would be expected to lead to improvements in mycoplasma detection, particularly for slow growing species from clinical specimens subject to contamination by commensal mycoplasmas.

Mycoplasmas have been differentiated on the basis of inhibitor tolerance. *Mycoplasma salivarium* was distinguished from *Mycoplasma orale* by its ability to grow on medium containing 0.2 mM $MnCl_2$ and *Mycoplasma gallisepticum* was distinguished from certain avain species by its tolerance to Cu^{++} (Dezewart *et al.*, 1991; Watanabe, 1994). In mycoplasmas, energy-generating mechanisms are diverse, and one possible approach to the development of selective media for specific *Mycoplasma* groups is the inhibition of

particular routes to ATP synthesis. The media can be made selective (for non-fermentative and non-arginine-hydrolysing mycoplasmas) by the addition of inhibitors like α-methyl glucoside, citrulline, lysine, ornithine and penicillin.

7.2 Study of pathogenicity factors

The small genome size of mollicutes and, particularly mycoplasmas, precludes their possession of the extensive metabolic activities present in other bacterial groups. Catabolic activities primarily associated with energy generation, rather than the provision of substrates for synthestic pathways and anabolism, are largely dependent upon extracellular sources of amino acids, nucleic acid precursors and lipids. The demonstrated energy-generating pathways of mollicutes produce low ATP yields and mycoplasma growth will generate relatively large quantities of metabolic end products and may deplete host tissues of substrates. One mechanism is arginine depletion, which is of particular importance in pathogenesis. Furthermore close physical association between mollicutes and host cells will enhance the potential significance of ammonia production from arginine and urea hydrolysis. The second most important mechanism is H_2O_2 production and superoxide formation during carbohydrate metabolism.

Arginine depletion is known to lead to cell growth and cell death (Claesson *et al.*, 1990) and production of ammonia by mycoplasmas may also be significant (Matsuura *et al.*, 1990). Arginine is a semi-essential nutrient for mammalian cells and known to be the first amino acid that is depleted by normal cell metabolism in culture (Wheatley *et al.*, 2000). Deprivation of arginine therefore disrupts many biochemical pathways and induces detrimental pleotypic responses. This leads to the quicker death of tumour cells compared to the depletion of other single essential amino acid (Scott *et al.*, 2000). The role of arginine hydrolysis in mycoplasmas able to use additional energy sources (glucose or organic acids) is also very interesting. It is possible that arginine is hydrolysed only to obtain energy. Ammonia, lactate and H_2O_2 may act as pathogenicity factors, and these toxic products are produced during substrate metabolism. Tryon and Baseman (1992) reported that H_2O_2, the product of both GP and NADH oxidase activities by mycoplasmas, causes oxidative damage to host cells. Thus the ability to oxidise glycerol may be a pathogenicity factor. NADH oxidase is present in all mycoplasmas; however production of

H_2O_2 by this enzyme will be dependent upon the net production of NADH during metabolism, which in turn requires that the mycoplasma is able to oxidise organic acids and/or sugars to acetate plus CO_2. However, ammonia from arginine hydrolysis may also produce toxic effects *in vivo* (Matsuura *et al.*, 1990). The production of H_2O_2 and other reactive oxygen species can cause lysis of red blood cells by a variety of *Mycoplasma* species and inhibition of ciliary movements by *M. mycoides* and *M. ovipneumoniae* in tracheal organ cultures (Tryon and Baseman, 1992; Niang *et al.*, 1998). H_2O_2 can be highly toxic to mammalian cells and the excretion of H_2O_2 has been implicated in the pathogenesis of lesions in the mucous membranes of the respiratory tract (Cohen and Somerson, 1967; Bradley and Erickson, 1981). The study of nucleases produced by *M. penetrans* clearly indicated that mycoplasma nucleases could act as virulence factors (Bendjennat *et al.*, 1999). A thiol peroxidase gene in *M. pulmonis* identified by Chambaud *et al.* (2001) is of interest because the production of H_2O_2 has also been suggested to be a virulence factor during *M. pulmonis* infections (Brennan and Feistein, 1969). However thiol peroxidase enzyme was not deduced from the genomes of other mollicutes.

Nicholas *et al.* (1996) have reported that European strains appear less virulent compared to African and Australian strains. Pini *et al.* (1999) have also shown similar results from experimental infection. European strains were unable to oxidise GP and did not produce H_2O_2. The variation of H_2O_2 production from NADH oxidation by all the strains studied in this project is very significant. It is possible that the ability to produce high levels of H_2O_2 from NADH oxidation is more widespread amongst pathogenic mycoplasmas, but this ability is rapidly selected against *in vitro* due to the effect of autonomously produced H_2O_2. *Mycoplasma* strains are more virulent when they are freshly isolated and their repeated passage *in vitro* may reduce their virulence. *M. bovis* strain 119B96 produced one mol of H_2O_2 per mol of oxygen taken up and might be more virulent. This strain was shown to produce reduced levels of H_2O_2 after *in vitro* repeated passage. It is possible that this strain might have lost NADH oxidase activity. Several studies have demonstrated that increasing *in vitro* passage reduces the virulence of *Mycoplasma hyopneumoniae*. Experimental challenge of pigs with low passage strain of *M. hyopneumoniae* induced lung lesions, while inoculation with high passage J strain fail to cause disease (Tajima andYagihashi, 1982; Zielinski and Ross, 1990). Low passage strain of *M. hyopneumoniae* specifically attack cilia in porcine tracheal organs, inducing ciliostasis and loss of cilia but attempts to

reproduce these pathogenic effects using strains of *M. hyopneumoniae* which had undergone increasing *in vitro* passage either failed or were significantly diminished (DeBey and Ross, 1994). Benedicte *et al.* (2002) have shown that the adherence of lamb synovial cells by *M. agalactiae* decreased with higher passage levels. It has recently been shown that a cytadherence molecule Gap A is missing in *Mycoplasma gallisepticum* strain R (high passage) (Papazisi *et al.*, 2000). Lin and Kleven (1984) reported that *in vitro* passage of *M. gallisepticum* strain affects its virulence. So it might be possible that high passage strain of *M. bovis* is attenuated and less pathogenic. However only experimental infection in calves will elucidate this.

7.3 Correlation of biochemical studies with the RFLP, PFGE, restriction endonucleases, SDS-PAGE and immunoblotting.

The biochemical data presented in this thesis showed generally uniform patterns of substrate oxidation between and within non-fermentative and non-arginine hydrolyzing mycoplasmas. *M. bovis* is a most important species in ruminants and is a cause of great economic losses in worldwide including the UK (Erno and Perreau, 1985; Nicholas *et al.*, 2002; Nicholas and Ayling, 2003). Few countries are free of disease because of the worldwide movement of cattle. In Europe *M. bovis* is believed to be responsible for 25-33% of outbreaks of calf pneumonia and control is largely restricted to management practices such as improving ventilation or reducing stocking densities and chemotherapy. Clinical and pathological signs are not characteristics for *M. bovis* so laboratory diagnosis is necessary for identification. A sandwich ELISA for *M. bovis* in which specific monoclonal antibodies, fixed to the microplate, captured *M. bovis* antigen from the medium was developed by Ball and Findlay (1998). *M. bovis* can be easily outgrown by other opportunistic mycoplasmas such as *M. bovirhinis* and acholeplasmas and occasionally antigenic variability of strains may make serological procedures unreliable. For these situations, more reliable tests need to be developed. The molecular study was mostly carried out within this species and biochemical data was compared with the molecular studies. This study mainly concerns correlation of biochemical studies with different molecular techniques such as RFLP, PFGE, RE, SDS-PAGE and immunoblotting. The study also concerned the critical evaluation of the above mentioned techniques for the diagnosis of *M. bovis*.

The results of RFLP and SDS-PAGE were consistent and all *M. bovis* strains showed identical banding patterns except *M. bovis* strain 119B96 (a multi-passage strain). The *uvr*C gene is species specific and well conserved within *M. bovis* and it is an ideal target gene for PCR based identification of *M. bovis*. One-dimensional SDS-PAGE is capable to detect only a minor proportion of the cell proteins nevertheless, protein patterns of *M. bovis* isolates can be essential for identification of strains and interspecies comparison of protein composition. Whole genomic DNA digestion of *Hind* III and *EcoR* I showed multiple cleavage sites so it was not possible to determine differences on the basis of this study. Similar findings were observed by Feenstra *et al.* (1999) with the *Hind* III restriction enzyme. The choice of methods used in epidemiological investigations depends on their reproducibility and on the organism tested. Various genetic techniques are currently used to compare *Mycoplasma* strains. Digestion of whole genomic DNA is often used but results are difficult to analyse.

All *M. bovis* strains were analysed by PFGE and immunoblotting, which showed discriminating banding patterns, although some strains were shown to be similar. Some *M. bovis* strains showed faint bands by PFGE because partial degradation of genomic DNA occurred during processing. Genomic heterogeneity has been seen among strains of *M. bovis* and it is common among *Mycoplasma* species and might be the result of their rapid evolution (Razin *et al.*, 1983). In the context of this study, *M. bovis* appeared to be genetically and antigenically variable. This variability could result either from very isolated enzootic areas or from different genetic determinisms. PFGE separates large DNA fragments and provides a new approach to molecular typing of mycoplasmas. Marois *et al.* (2001b) have recently used PFGE for differentiation of *M. gallisepticum* and *M. imitans* and reported PFGE as unique and discriminatory. This technique had been used to construct a physical map of the *M. agalactiae* (Tola *et al.*, 2001). The assessment of molecular techniques showed that immunoblotting and PFGE are the most robust and discriminatory, and can be useful tools for the identification and molecular typing of *M. bovis*. It was confirmed that the *M. bovis* population is genetically diverse. However, there is still no explanation for the fact that, on the one hand, this mycoplasma causes severe disease in different organs and tissues whereas, on the other hand, it is often detected in animals lacking any clinical signs. This apparent discrepancy suggests the prevalence of a considerable degree of heterogeneity among population of this organism.

7.4 Future work

Biochemical tests are very important in reducing the range of serological and/or molecular tests needed to identify target organisms. Biochemical tests might also be useful where there are serological cross-reactions. For the development of rapid biochemical tests a comprehensive knowledge of pattern and kinetics of substrate oxidation amongst species is required. It is possible that biochemical testing procedures might also prove useful in the identification of mycoplasmas from other groups of host species. Rice *et al.* (2000) have developed a test based on hydrolysis of the chromogenic substrate α-glucosidase (maltase) substrate (p-nitrophenyl-α-D-glucopyranoside α-glucosidase, pNPG, colourless) to give a brightly coloured product (p-nitrophenol, yellow). The chromogenic substrate 3.3-diaminobenzidine (DAB) has also been used for the detection of H_2O_2 (Rice *et al.*, 2001). The possession of α-glucosidase activity (maltose oxidising ability) distinguishes *M. mycoides* subsp. *mycoides* SC strains from subsp. *mycoides* LC and subsp. *capri* strains.

The emphasis of the work described in this thesis concerns the more applied aspects of mycoplasmology relating to strain characterisation and identification on which a great deal of fundamental biochemical data on substrate utilisation has been obtained. *M. genitalium* and *M. pneumoniae* genomes have been completely sequenced (Fraser *et al.*, 1995; Himmelreich *et al.*, 1996). Glass *et al.* (2000) have completely sequenced *Ureaplasma urealyticum,* the third mycoplasma to be sequenced. It would be expected that where genes are identified from sequence data, the corresponding metabolic activities could be found and for each distinct metabolic activity the corresponding gene would be present, however, this is not always the case. For example, Pollack *et al.* (1997) suggested that in *M. genitalium* and *M. pneumoniae*, the same enzyme might carry out lactate and malate dehydrogenase activities, whereas in cell-walled bacteria, these activities are independent. This is consistent with the evolutionary trend of mycoplasmas towards a smaller genome (Razin *et al.*, 1998). Mycoplasmas also appear to use transport proteins with wide substrate specificity. Despite the fact that mycoplasmas are dependent upon extracellular sources of a diverse range of amino acids, nucleic acids precursors, vitamins and other compounds, the *M. genitalium* genome contains only 34 transport proteins (Himmelreich *et al.*, 1997). It is also possible that the possession of genes for linked enzyme activities, which might constitute a pathway, does not necessarily mean that the pathway indicated is

functional. For example, in *M. pneumoniae*, genes for the arginine dihydrolase pathway are present, but this species does not hydrolyse arginine (Himmelreich *et al.*, 1996, 1997; Tully and Razin, 1996). Recently Chambaud *et al.* (2001) who sequenced the *M. pulmonis* genome, identified genes for malto-dextrin ABC transporter system, a dextrinase and α-amylase in this organism. The importance of biochemistry can be understood from the fact that they did not know the functions of these genes. Therefore it is very important to have a comprehensive knowledge of microbial physiology of mycoplasmas if interpretation of complete genome sequence data is to be realised.

The results obtained from pattern and kinetics of substrate utilisation were also of significance for medium development. Potential energy substrates have been identified for species tested which increase the growth yields but work on improving growth media still needs to be done. PRM medium should be further modified by replacing serum with fatty acids and attempts should also be made to find out whether yeast extract and fresh yeast extract both are necessary for this medium. Improved definition of cell production requires that continued effort be applied to the development of defined media and of minimal defined media. A rapid chromogenic substrate test has been developed for the identification of *M. agalactiae* and *M. bovis*. The application of chromogenic substrates to the detection of mycoplasmas would appear potentially important. This approach to biochemical characterisation is dependent upon range and availability of chromogenic substrates. The lipase purification, structure and gene sequence may also be attempted.

The metabolic pathways identified in these species should be correlated with the genome sequence. However a major goal for researchers in this area is to sequence the genome of *M. bovis*. Little is known of strain virulence, or of virulence factors such as adhesions or toxins. It is hoped that eventually the identification of the genes responsible for pathogenicity will enable the development of improved vaccines. However the comprehensive biochemical data obtained in this thesis will be helpful for the functions of identified genes. The function of isopropanol in non-fermentative and non-arginine hydrolysing species tested need to be studied in detail. In this study it was shown that the repeated *in vitro* passage of *M. bovis* resulted in the reduction of H_2O_2 to negligible levels and this might be due to deletion of NADH oxidase gene or altered regulatory function. Initially, the nucleotide sequences of the NADH oxidase gene from low and high H_2O_2

producing strains of *M. bovis* should be determined. One way of determining this would be to find out NADH oxidase gene sequence in completely sequenced mycoplasmas and primers may be designed from that sequence. Other experiments such as cytadherence of multi-passage strains may also be carried out. Attempts should also be made to study the pathogenicity of passage zero and high passage strains using animal models. It might be helpful for the screening and development of live vaccine strains before vaccinating calves. Antibiotic sensitivity tests of zero, medium and high passage strains may also be tried. Attempts should also be made to sequence the PCR-RFLP product of multi-passage and zero passage strains which might show some differences in base pair.

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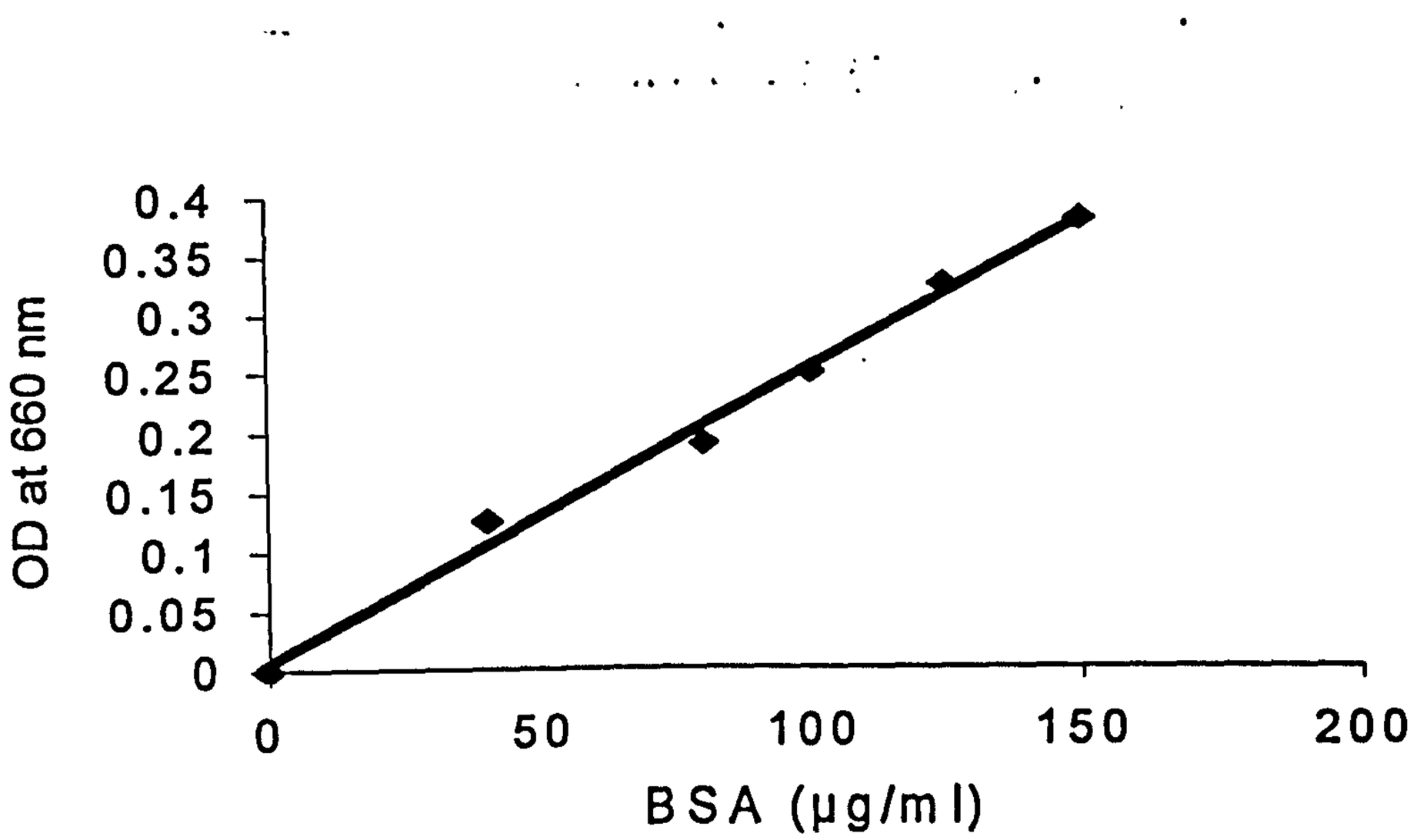
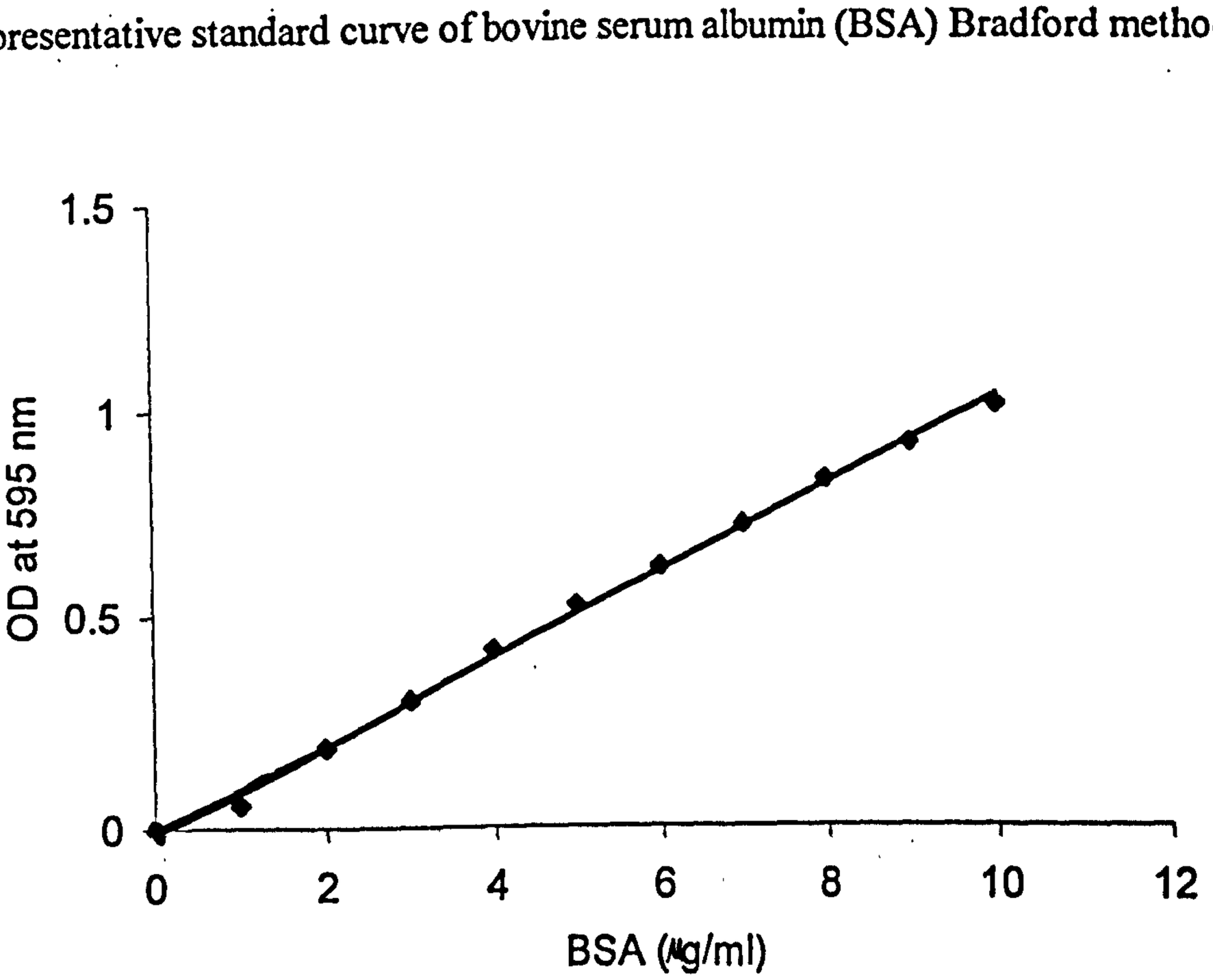
Appendix 1

Representative standard curve of bovine serum albumin (BSA) at 660 nm Markwell method (1978).

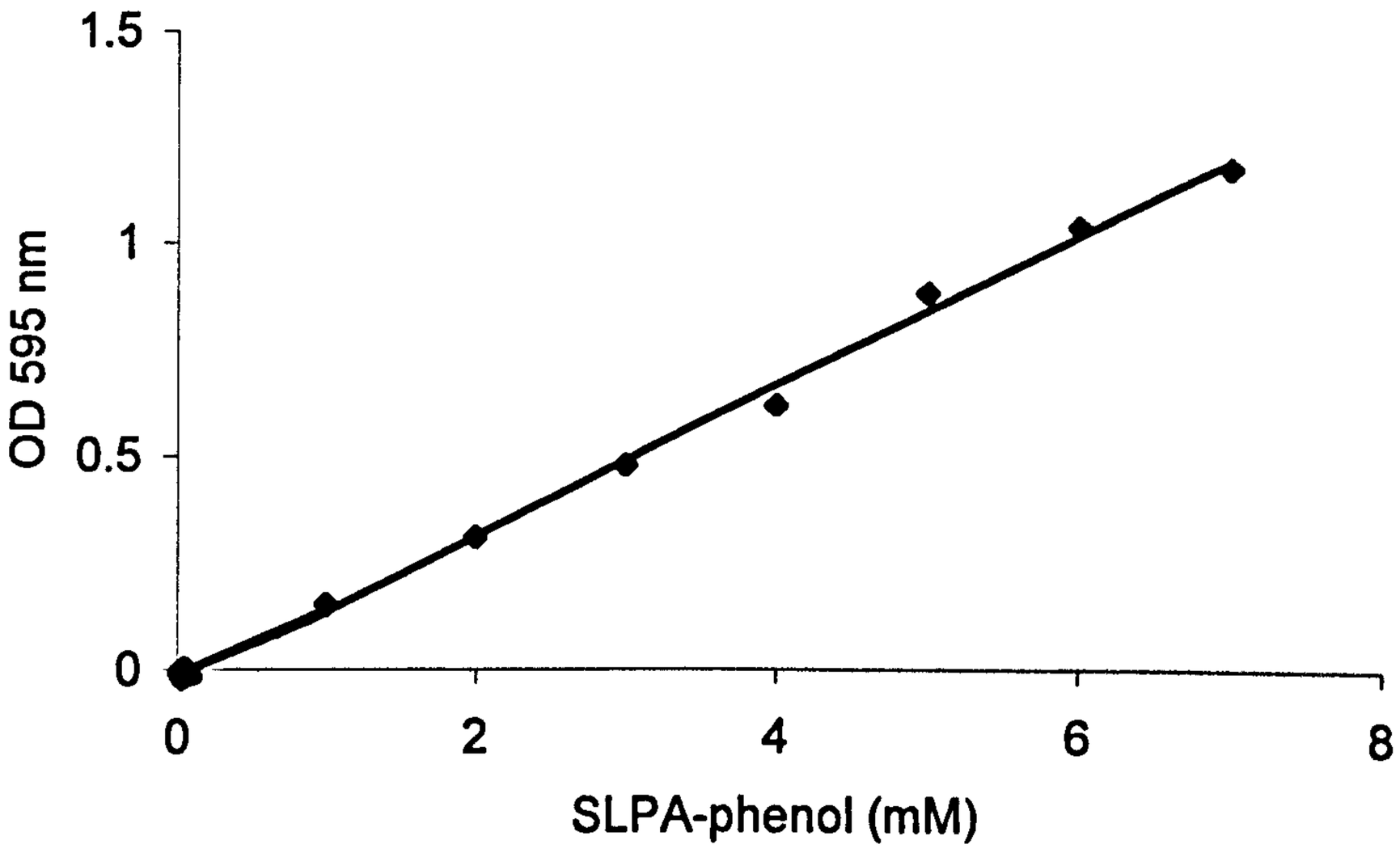


Representative standard curve of bovine serum albumin (BSA) Bradford method (1976).

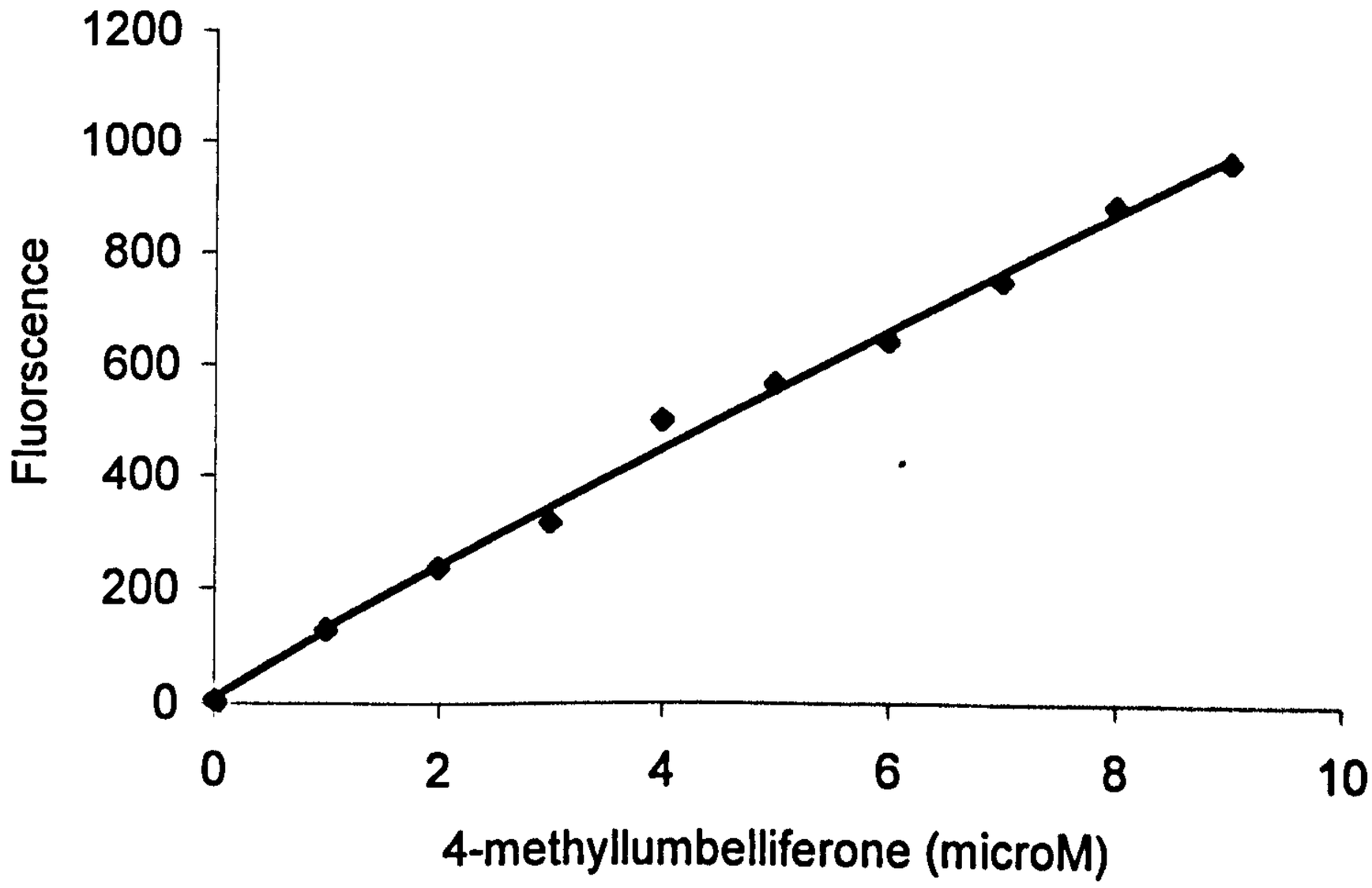


Appendix 2

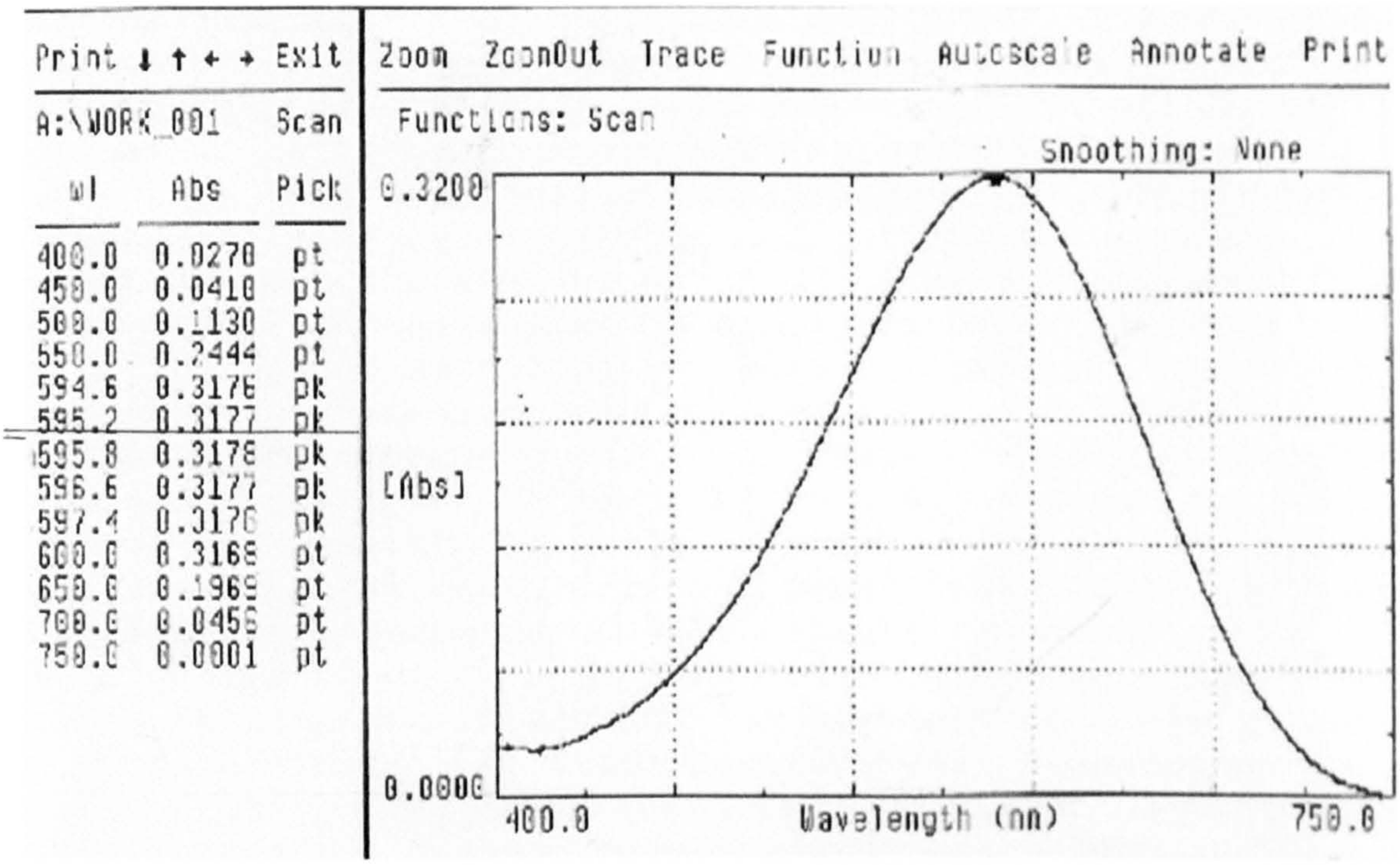
Representative standard curve of SLPA-phenol



Representative standard curve of 4-methyllumbelliferone



Absorption spectra of the SLPA-phenol 0.2 mM using scanning spectrophotometer (PU 8720 UV/VIS, Philips)



Appendix 3

Complete sequence of *uvr*C gene

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taacaaatttcaagtgttgcaaactccaacattttgcataattaaaaacaaaaacattt
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tctagttatttcaaagtcatctataactttgagatttttttaggtactgcttgtattc

Publications

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